

# Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US04/042701

International filing date: 17 December 2004 (17.12.2004)

Document type: Certified copy of priority document

Document details: Country/Office: US

Number: 60/530,480

Filing date: 17 December 2003 (17.12.2003)

Date of receipt at the International Bureau: 11 February 2005 (11.02.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)



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PROVISIONAL APPLICATION COVER SHEET

Transmitted herewith for filing under 37 CFR 1.53(c) is the provisional patent application of the following Inventor(s): A. Krishna PRASAD, ET AL;  
For: METHODS OF PRODUCING IMMUNOGENIC PEPTIDE CARRIER CONJUGATES

1. Papers enclosed which are required for filing date under 37 CFR 1.51(c) and 1.53 (c):

- Pages of specification – 78 pages
- Sequence Listing – 20 pages
- Pages of claims – 39 pages
- Page(s) of abstract – 1 page
- Sheet(s) of drawing – 9 sheets
- Formal
- Informal

2. Inventor(s) name(s) and address(es) (or list attorney docket number as alphanumeric identifier):

1. A. Krishna PRASAD 105 Worsham Drive, Chapel Hill, NC 27516  
2. Rasappa G. ARUMUGHAM 102 Barton Lane, Chapel Hill, NC 27516

3.

4.

5.

3. Assignment

An assignment of the invention to:  
Wyeth  
Five Giraldia Farms  
Madison, New Jersey 07940

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  - No
  - Yes, the name of the U.S. Government agency and the Government contract numbers are \_\_\_\_\_.



Daryl L. Webster  
Attorney for Applicants  
Reg. No. 34,276

Wyeth  
Patent Law Department  
Five Giralda Farms  
Madison, NJ 07940  
Tel. No. 845 602 4638

ii

**METHODS OF PRODUCING**  
**IMMUNOGENIC PEPTIDE CARRIER CONJUGATES**

The present invention is directed to methods of producing conjugates of peptide immunogens with protein/polypeptide carrier molecules, which are useful as 5 immunogens, wherein peptide immunogens are conjugated to protein carriers via activated functional groups on amino acid residues of the carrier or of the optionally attached linker molecule, and wherein any unconjugated reactive functional groups on amino acid residues are inactivated via capping, thus retaining the immunological functionality of the carrier molecule, but reducing the propensity for undesirable 10 reactions that could render the conjugate less safe or effective. Furthermore, the invention relates to such immunogenic products and immunogenic compositions containing such immunogenic products made by such methods.

**BACKGROUND OF THE INVENTION**

The essence of adaptive immunity is the ability of an organism to react to the 15 presence of foreign substances and produce components (antibodies and cells) capable of specifically interacting with and protecting the host from their invasion. An "antigen" or "immunogen" is a substance that is able to elicit this type of immune response and also is capable of interacting with the sensitized cells and antibodies that are manufactured against it.

20 Antigens or immunogens are usually macromolecules that contain distinct antigenic sites or "epitopes" that are recognized and interact with the various components of the immune system. They can exist as individual molecules composed of synthetic organic chemicals, proteins, lipoproteins, glycoproteins, RNA, DNA, or polysaccharides, or they may be parts of cellular structures (bacteria or fungi) or 25 viruses (Harlow and Lane 1988a,b, c; Male *et al.*, 1987).

Small molecules like short peptides, although normally able to interact with the products of an immune response, often cannot cause a response on their own. These peptide immunogens or "haptens" as they are also called, are actually 30 incomplete antigens, and, although not able by themselves to cause immunogenicity or to elicit antibody production, can be made immunogenic by coupling them to a

suitable carrier. Carriers typically are protein antigens of higher molecular weight that are able to cause an immunological response when administered *in vivo*.

In an immune response, antibodies are produced and secreted by the B-lymphocytes

5      in conjunction with the T-helper ( $T_H$ ) cells. In the majority of hapten-carrier systems, the B cells produce antibodies that are specific for both the hapten and the carrier. In these cases, the T lymphocytes will have specific binding domains on the carrier, but will not recognize the hapten alone. In a kind of synergism, the B and T cells cooperate to induce a hapten-specific antibody response. After such an immune

10     response has taken place, if the host is subsequently challenged with only the hapten, usually it will respond by producing hapten-specific antibodies from memory cells formed after the initial immunization.

Synthetic haptens mimicking some critical epitopic structures on larger

15     macromolecules are often conjugated to carriers to create an immune response to the larger "parent" molecule. For instance, short peptide segments can be synthesized from the known sequence of a protein and coupled to a carrier to induce immunogenicity toward the native protein. This type of synthetic approach to the immunogen production has become the basis of much of the current research into

20     the creation of vaccines. However, in many instances, merely creating a B-cell response by using synthetic peptide-carrier conjugates, however well designed, will not always guarantee complete protective immunity toward an intact antigen. The immune response generated by a short peptide epitope from a larger viral particle or bacterial cell may only be sufficient to generate memory at the B cell level. In these

25     cases it is generally now accepted that a cytotoxic T-cell response is a more important indicator of protective immunity. Designing peptide immunogens with the proper epitopic binding sites for both B-cell and T-cell recognition is one of the most challenging research areas in immunology today.

30     The approach to increasing immunogenicity of small or poorly immunogenic molecules by conjugating these molecules to large "carrier" molecules has been utilized successfully for decades (see, e.g., Goebel *et al.* (1939) *J. Exp. Med.* 69: 53). For example, many immunogenic compositions have been described in which

purified capsular polymers have been conjugated to carrier proteins to create more effective immunogenic compositions by exploiting this "carrier effect." Schneerson *et al.* (1984) *Infect. Immun.* 45: 582-591). Conjugation has also been shown to bypass the poor antibody response usually observed in infants when immunized with a free 5 polysaccharide (Anderson *et al.* (1985) *J. Pediatr.* 107: 346; Insel *et al.* (1986) *J. Exp. Med.* 158: 294).

Hapten-carrier conjugates have been successfully generated using various cross-linking/coupling reagents such as homobifunctional, heterobifunctional, or zero-length 10 cross linkers. Many such methods are currently available for coupling of saccharides, proteins, and peptides to peptide carriers. Most methods create amine, amide, urethane, isothiourea, or disulfide bonds, or in some cases thioethers. A disadvantage to the use of coupling reagents, which introduce reactive sites into the side chains of reactive amino acid molecules on carrier and/or hapten molecules, is 15 that the reactive sites if not neutralized are free to react with any unwanted molecule either *in vitro* (thus adversely affecting the functionality or stability of the conjugate(s)) or *in vivo* (thus posing a potential risk of adverse events in persons or animals immunized with the preparations). Such excess reactive sites can be reacted or "capped", so as to inactivate these sites, utilizing various known chemical reactions, 20 but these reactions may be otherwise disruptive to the functionality of the conjugates. This may be particularly problematic when attempting to create a conjugate by introducing the reactive sites into the carrier molecule, as its larger size and more complex structure (relative to the hapten) may render it more vulnerable to the disruptive effects of chemical treatment. In fact, no examples are known of methods 25 whereby a conjugate is made by first activating the carrier, then reacting with the hapten in a conjugation reaction, and finally "capping" the remaining reactive sites, while preserving the ability of the resulting conjugate to function as an immunogenic composition having the desired properties of the "carrier effect".

SUMMARY OF THE INVENTION

The present invention is directed to methods of producing an immunogenic conjugate of a peptide immunogen with a protein/polypeptide carrier, wherein the peptide immunogen is conjugated to the carrier via derivatized functional groups of amino acid residues of the carrier such as lysine residues, and wherein any unconjugated, derivatized functional groups of the amino acid residues are inactivated via capping to block them from reacting with other molecules, including proteins/polypeptides thereby preserving the functionality of the carrier, such that it retains its ability to elicit the desired immune responses against the peptide immunogen that would otherwise not occur without a carrier. Furthermore, the invention also relates to conjugates produced by the above methods, and to immunogenic compositions containing such conjugates.

In one embodiment, the invention is directed to a first method for conjugating a peptide immunogen via a reactive group of an amino acid residue of the peptide immunogen to a protein/polypeptide carrier having one or more functional groups, the method comprising the steps of: (a) derivatizing one or more of the functional groups of the protein/polypeptide carrier to generate a derivatized molecule with reactive sites; (b) reacting the derivatized protein/polypeptide carrier of step (a) with a reactive group of an amino acid residue of the peptide immunogen under reaction conditions such that the peptide immunogen is conjugated to the derivatized protein/polypeptide carrier via the functional groups; and (c) further reacting the conjugate with a capping reagent to inactivate free, reactive functional groups on the activated protein/polypeptide carrier, thereby preserving the functionality of the carrier against the peptide immunogen that would otherwise not occur without a carrier.

In one embodiment, the protein/polypeptide carrier is selected from the group consisting of human serum albumin, keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, influenza hemagglutinin, PAN-DR binding peptide (PADRE polypeptide), malaria circumsporozite (CS) protein, hepatitis B surface antigen (HB<sub>s</sub>Ag<sub>19-28</sub>), Heat Shock Protein (HSP) 65, *Bacillus Calmette-Guerin* (BCG), cholera toxin, cholera toxin mutants with reduced toxicity, diphtheria toxin, CRM<sub>197</sub> protein that is cross-reactive with diphtheria toxin, recombinant Streptococcal

C5a peptidase, *Streptococcus pyogenes* ORF1224, *Streptococcus pyogenes* ORF1664, *Streptococcus pyogenes* ORF 2452, *Chlamydia pneumoniae* ORF T367, *Chlamydia pneumoniae* ORF T858, Tetanus toxoid, HIV gp120 T1, microbial surface components recognizing adhesive matrix molecules (MSCRAMMS), growth factor / 5 hormone, cytokines and chemokines.

In another embodiment, the protein/polypeptide carrier contains a T-cell epitope.

In yet another embodiment, the protein/polypeptide carrier is a bacterial toxoid such 10 as a tetanus toxoid, cholera toxin or cholera toxin mutant as described above. In a preferred embodiment, the protein/polypeptide carrier is CRM<sub>197</sub>.

In still yet another embodiment, the protein/polypeptide carrier may be an influenza 15 hemagglutinin, a PADRE polypeptide, a malaria CS protein, a Hepatitis B surface antigen (HSBAg<sub>19-28</sub>), a heat shock protein 65 (HSP 65), or a polypeptide from *Mycobacterium tuberculosis* (BCG).

In a preferred embodiment, the protein/polypeptide carrier is selected from Streptococcal rC5a peptidase, *Streptococcus pyogenes* ORF1224, *Streptococcus pyogenes* ORF1664 or *Streptococcus pyogenes* ORF2452, *Chlamydia pneumoniae* ORF T367, and *Chlamydia pneumoniae* ORF T858. 20

In one embodiment, protein/polypeptide carrier is a growth factor or hormone, which 25 stimulates or enhances immune response and is selected from the group consisting of IL-1, IL-2,  $\gamma$ -interferon, IL-10, GM-CSF, MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES.

In one embodiment, the peptide immunogen is selected from a bacterial protein, a viral protein, and a eukaryotic protein.

In another embodiment, the peptide immunogen is derived from a bacterial protein 30 antigen from *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Escherichia coli*, *Klebsiella enterobacter*, *Listeria monocytogenes*, *Vibrio cholerae*, *Clostridium perfringens*, *Clostridium botulinum*, *Pseudomonas* species, *Salmonella*

*typhimurium*, *Borrelia burgdorferi*, *Shigella flexneri*, *Shigella boydii*, *Shigella dysenteriae*, *Alloiococcus otitidis* and Group B streptococci.

In another embodiment, the peptide immunogen is derived from a protein antigen

5 5 from a virus selected from the group consisting of human immunodeficiency virus (HIV), herpes simplex virus (HSV), human papilloma virus (HPV), parainfluenza virus (PIV), vesicular stomatitis virus (VSV), respiratory syncytial virus (RSV), Epstein-Barr virus (EBV), coronavirus, vaccinia virus, rotavirus, rabies virus, hepatitis C virus (HCV) and hepatitis B virus (HBV).

10 In yet another embodiment, the peptide immunogen is derived from a protein antigen from a fungus selected from a *Candida* species, a *Cryptococcus* species, a *Coccidioides* species, a *Histoplasma* species, and an *Aspergillus* species.

15 In another embodiment, the peptide immunogen is derived from a protein antigen from a parasite selected from a *Plasmodium*, a *Trypanosome*, a *Schistosome*, and a *Leishmania*.

20 In another embodiment, the peptide immunogen is derived from a protein antigen from a eukaryote. In a preferred embodiment, the eukaryote is a human.

In yet another preferred embodiment, the peptide immunogen from the human is derived from a malignant tumor. In a more preferred embodiment, the peptide immunogen is from a tumor antigen from a renal cell carcinoma, a breast carcinoma,

25 25 a melanoma and a prostate carcinoma. In another preferred embodiment, the peptide antigen is derived from the tumor antigen, carcinoembryonic antigen (CEA).

In one aspect, the invention provides a peptide immunogen comprising A $\beta$  peptide or fragments of A $\beta$  or analogs thereof eliciting an immunogenic response against

30 30 certain epitopes within A $\beta$ . Immunogenic peptides of the invention include immunogenic heterologous peptides. In some immunogenic peptides, an A $\beta$  fragment is linked to a carrier to form an immunogenic heterologous peptide, and

then this heterologous peptide is linked to a carrier using a method of the present invention to form a conjugate.

In another aspect of the invention, the peptide immunogen is a polypeptide comprising an N-terminal segment of at least residues 1-5 of A $\beta$ , the first residue of

- 5 A $\beta$  being the N-terminal residue of the polypeptide, wherein the polypeptide is free of a C-terminal segment of A $\beta$ . In yet another aspect of the invention, the peptide immunogen is a polypeptide comprising an N-terminal segment of A $\beta$ , the segment beginning at residue 1-3 of A $\beta$  and ending at residues 7-11 of A $\beta$ . In some aspects of the invention, the peptide immunogen is an agent that induces an immunogenic
- 10 response against an N-terminal segment of A $\beta$ , the segment beginning at residue 1-3 of A $\beta$  and ending at residues 7-11 of A $\beta$  without inducing an immunogenic response against an epitope within residues 12-43 of A $\beta$ 43. In another aspect of the invention, the peptide immunogen is a heterologous polypeptide comprising a segment of A $\beta$  linked to a heterologous amino acid sequence that induces a helper T-cell response
- 15 against the heterologous amino acid sequence and thereby a B-cell response against the N-terminal segment.

In some peptide immunogens, the N-terminal segment of A $\beta$  is linked at its C-terminus to a heterologous polypeptide. In some peptide immunogens, the N-terminal segment of A $\beta$  is linked at its N-terminus to a heterologous polypeptide. In

- 20 some peptide immunogens, the N-terminal segment of A $\beta$  is linked at its N and C termini to first and second heterologous polypeptides. In some peptide immunogens, the N-terminal segment of A $\beta$  is linked at its N terminus to a heterologous polypeptide, and at its C-terminus to at least one additional copy of the N-terminal segment. In some peptide immunogens, the polypeptide comprises from N-terminus
- 25 to C-terminus, the N-terminal segment of A $\beta$ , a plurality of additional copies of the N-terminal segment, and the heterologous amino acid segment.

In some of the above peptide immunogens, the polypeptide further comprises at least one additional copy of the N-terminal segment. In some of the above peptide immunogens, the fragment is free of at least the 5 C-terminal amino acids in A $\beta$ 43.

- 30 In some aspects of the above peptide immunogens, the fragment comprises up to 10 contiguous amino acids from A $\beta$ .

In another aspect, the invention provides a peptide immunogen comprising A $\beta$  peptide or fragments of A $\beta$  or analogs thereof eliciting an immunogenic response against certain epitopes within A $\beta$  may be in a configuration referred to as a multiple 5 antigenic peptide (MAP) configuration.

In some of the above aspects of the invention, the peptide immunogen from the N-terminal half of A $\beta$ . In some aspects of the invention, the peptide immunogen is an A $\beta$  fragment selected from the group consisting of A $\beta$ 1-3, 1-4, 1-5, 1-6, 1-7, 1-10, 1-10, 11-12, 1-16, 3-6, and 3-7. In some of the above aspects of the invention, the peptide immunogen is from the internal region of A $\beta$ . In some aspects of the invention, the peptide immunogen is an A $\beta$  fragment selected from the group consisting of A $\beta$ 13-28, 15-24, 17-28, and 25-35. In some of the above aspects of the invention, the peptide immunogen from the C-terminal end of A $\beta$ . In some aspects of the invention, the peptide immunogen is an A $\beta$  fragment selected from the group consisting of A $\beta$ 33-42, 35-40, and 35-42. In some aspects of the invention, the peptide immunogen is an A $\beta$  fragment selected from the group consisting of A $\beta$ 1-3, 1-4, 1-5, 1-6, 1-7, 1-10, 1-11, 1-12, 1-16, 1-28 3-6, 3-7, 13-28, 15-24, 17-28, 25-35, 33-42, 35-40, and 35-42. In some aspects of the invention, the peptide immunogen 15 is an A $\beta$  fragment selected from the group consisting of A $\beta$ 1-5, A $\beta$ 1-7, A $\beta$ 1-9, and A $\beta$ 1-12. In some aspects of the invention, the peptide immunogen is an A $\beta$  fragment selected from the group consisting of A $\beta$ 1-5-L, A $\beta$ 1-7-L, A $\beta$ 1-9-L, and A $\beta$ 1-12-L, where L is a linker. In some aspects of the invention, the peptide immunogen is an A $\beta$  fragment selected from the group consisting of A $\beta$ 1-5-L-C, A $\beta$ 1-7-L-C, A $\beta$ 1-9-L-C, 20 and A $\beta$ 1-12-L-C, where C is a cysteine amino acid residue.

In some of the above peptide immunogens, the heterologous polypeptide is selected from the group consisting of peptides having a T-cell epitope, a B-cell epitope and combinations thereof.

In one embodiment, the functional group of one or more amino acid molecules of the 30 protein/polypeptide carrier or of the optionally attached polypeptide linker is derivatized using a cross-linking reagent. In another embodiment, the derivatizing reagent is a zero-length cross-linking reagent. In another embodiment, the

derivatizing reagent is a homobifunctional cross-linking reagent. In yet another embodiment, the derivatizing reagent is a heterobifunctional cross-linking reagent.

In a preferred embodiment, the heterobifunctional reagent is a reagent that reacts

- 5 with a primary or a  $\epsilon$ -amine functional group of one or more amino acid molecules of the protein/polypeptide carrier and a pendant thiol group of one or more amino acid molecules of the peptide immunogen. In one embodiment, the heterobifunctional reagent is N-succinimidyl bromoacetate.
- 10 In another embodiment, the primary or  $\epsilon$ -amine functional group is lysine. In yet another embodiment, the derivatization of the primary or  $\epsilon$ -amine functional group of the lysine of the protein/polypeptide carrier with N-succinimidyl bromoacetate results in the bromoacetylation of the primary or  $\epsilon$ -amine residues on lysine molecules on the protein/polypeptide carrier. In a more preferred embodiment, the pendant thiol
- 15 group is a cysteine residue of the peptide immunogen, which may be localized at the amino-terminus of the peptide immunogen, at the carboxy-terminus of the peptide immunogen or internally in the peptide immunogen.

In another embodiment, the pendant thiol group is generated by a thiolating reagent

- 20 such as N-acetyl homocysteinethio lactone, Traut's reagent (2-iminothilane) SATA (N-Succinimidyl S-acetylthioacetate), SMPT (4-Succinimidyl oxycarbonyl-methyl2-pyridylthio toluene), Sulfo LC SPDP (Sulfo Succinimidyl pyridyl dithio propionamido hexanoate), SPDP (Succinimidyl pyridyl dithio propionate). In a preferred embodiment, the capping reagent that is used to inactivate free reactive,
- 25 functional groups on the activated protein/polypeptide carrier is selected from the reagent group consisting of cysteamine, N-acetylcysteamine, and ethanolamine. In a particularly preferred embodiment, the capping reagent that is used to inactivate free reactive functional groups on the activated protein/polypeptide carrier is selected from the reagent group consisting of sodium hydroxide, sodium carbonate, ammonium bicarbonate and ammonia.
- 30

In one embodiment, the reactive group of the amino acid residue of the peptide immunogen is a free sulfhydryl group.

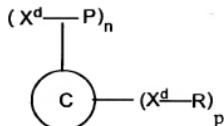
In another embodiment, one or more of the functional groups are on a linker, which is optionally attached to the protein/polypeptide carrier. In a preferred embodiment, the linker is a peptide linker. In a more preferred embodiment, the peptide linker is 5 polylysine.

In another embodiment, the invention is directed to a second method for conjugating a peptide immunogen of a protein/polypeptide with a protein/polypeptide carrier having the structure:

10

wherein,

C is a protein/polypeptide carrier and X is a derivatizable functional group of an amino acid residue on the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to the protein/polypeptide carrier, 15 and wherein m is an integer greater than 0, but less than or equal to 85, the method comprising the steps of: (a) derivatizing one or more of the functional groups of the protein/polypeptide carrier or of the optionally attached linker molecule to generate a derivatized molecule with reactive sites; (b) reacting the derivatized protein/polypeptide carrier of step (a) with a reactive group of an amino acid residue 20 of the peptide immunogen to form a covalently coupled peptide immunogen-protein/polypeptide carrier conjugate; and (c) further reacting the said conjugate with a capping reagent to inactive the free reactive functional groups on the activated protein/polypeptide carrier, such that the capped groups are not free to react with other molecules, including proteins/polypeptides thereby preserving the functionality 25 of the carrier, such that it retains its ability to elicit the desired immune responses against the peptide immunogen that would otherwise not occur without a carrier so as to generate a capped peptide immunogen-protein/polypeptide carrier conjugate having the formula:



wherein,

5 C is the protein/polypeptide carrier and  $X^d$  is a derivatized functional group of an amino acid residue of the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to the protein/polypeptide carrier, and, wherein,

10 P is the peptide immunogen molecule covalently attached to the derivatized functional group on the amino acid residue on the protein carrier or optionally on an amino acid residue on a peptide linker covalently attached to a protein/polypeptide carrier, R is a capping molecule covalently attached to the derivatized functional group on an amino acid residue on the protein/polypeptide carrier or optionally on an amino acid residue on a peptide linker covalently attached to a protein/polypeptide carrier, n is an integer greater than 0, but less than or equal to 85, and p is an integer greater than 0, but less than 85.

15

The detailed embodiments for the first method described above are also applicable to the conjugates just described prepared by the second method.

20 In one embodiment, the invention is directed to peptide immunogen-protein/polypeptide carrier conjugates wherein the protein/polypeptide carrier has the formula:

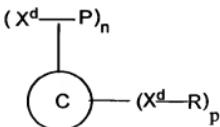


wherein,

25 C is a protein/polypeptide carrier and X is a derivatizable functional group of an amino acid residue on the protein/polypeptide carrier or optionally of an amino

acid residue of a peptide linker covalently attached to the protein/polypeptide carrier, and, wherein, m is an integer greater than 0, but less than or equal to 85, and wherein the capped peptide immunogen-protein/polypeptide carrier conjugate has the formula:

5



wherein,

C is the protein/polypeptide carrier and  $X^d$  is a derivatized functional group of an amino acid residue of the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to the protein/polypeptide carrier, and,

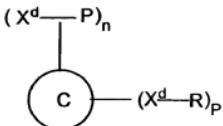
10 wherein, P is the peptide immunogen molecule covalently attached to the derivatized functional group of the amino acid residue of the protein carrier or optionally of an amino acid residue of a peptide linker covalently attached to a protein/polypeptide carrier, R is a capping molecule covalently attached to the derivatized functional group of an amino acid residue of the protein/polypeptide carrier or optionally of an

15 amino acid residue of a peptide linker covalently attached to a protein/polypeptide carrier, thereby preserving the functionality of the carrier, such that it retains its ability to elicit the desired immune responses against the peptide immunogen that would otherwise not occur without a carrier., n is an integer greater than 0, but less than or equal to 85, and p is an integer greater than 0, but less than 85.

20 The detailed embodiments for the first and second methods described above are also applicable to the conjugates just described.

In another embodiment, the invention is directed to peptide immunogen-protein/polypeptide carrier conjugates generated according to the second method of

25 the invention and having the formula:



wherein,

C is the protein/polypeptide carrier and  $X^d$  is a derivatized functional group of an amino acid residue of the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to the protein/polypeptide carrier, and,

5 wherein, P is the peptide immunogen molecule covalently attached to the derivatized functional group of the amino acid residue of the protein carrier or optionally of an amino acid residue of a peptide linker covalently attached to a protein/polypeptide carrier, R is a capping molecule covalently attached to the derivatized functional

10 group of an amino acid residue of the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to a protein/polypeptide carrier thereby preserving the functionality of the carrier, such that it retains its ability to elicit the desired immune responses against the peptide immunogen that would otherwise not occur without a carrier., n is an integer greater than 0, but less than or

15 equal to 85, and p is an integer greater than 0, but less than 85.

The detailed embodiments for the second method described above are also applicable to the conjugates generated by the second method, as just described.

20 In another embodiment, the invention is directed to immunogenic compositions comprising a conjugate of a peptide immunogen with a protein/polypeptide carrier generated by the second method of the invention, together with one or more pharmaceutically acceptable excipients, diluents, and adjuvants.

25 The detailed embodiments for the second method and the conjugates generated thereby described above are also applicable to immunogenic compositions containing those conjugates as just described.

In another embodiment, the invention is directed to a method for inducing an immune response in a mammalian subject, which comprises administering an effective amount of an immunogenic composition of the present invention to the subject.

- 5 The detailed embodiments applicable to the immunogenic composition containing the conjugates of the present invention are also applicable to the embodiment of the invention directed to the method of use of these immunogenic compositions.

**BRIEF DESCRIPTION OF FIGURES**

Figure 1: Flow chart depicting the process chemistry used for conjugation of A $\beta$  peptide fragments to protein/polypeptide carrier CRM<sub>197</sub>.

5    Figure 2: Flow chart depicting acid hydrolysis chemistry used for quantitative determination of S-carboxymethylcysteine and S-carboxymethylcysteamine as evaluation of the degree of conjugation of peptide immunogen-protein/polypeptide conjugates.

Figure 3: This figure depicts the pH dependence of the conjugation reaction.

10   Figure 4: This figure depicts the dependence of A $\beta$ -peptide/CRM conjugation on peptide: CRM ratio.

Figure 5: Verification of capping process for A $\beta$ 1-7/CRM conjugation. The pH of the reaction was 9.15. Reaction time with peptide was 16 hrs, capping with *N*-acetylcysteamine was 8 hrs.

15   Figure 6: Conjugation and capping with various peptide: CRM ratios with peptide . The pH of the reaction was 9.0. Reaction time with peptide was 16 hrs, capping with *N*-acetylcysteamine was 8 hrs.

Figure 7: Day 36 titers of primate sera following immunization of primates with A $\beta$  peptide conjugates with various adjuvants.

20   Figure 8: Day 64 titers of primate sera following immunization of primates with A $\beta$ -peptide conjugates with various adjuvants.

Figure 9: Primate titers by day and treatment group. Primates were immunized with A $\beta$  1-7 or A $\beta$  1-5 CRM<sub>197</sub> conjugates with alum or RC529 as adjuvants and titers of anti-A $\beta$  antibodies were measured at day 29, 36, 57 and 54.

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**BRIEF DESCRIPTION OF SEQUENCES**

SEQ ID NO:	Sequence	Description
1	DAEFR-C	A $\beta$ 1-5-C
2	DAEFRHD-C	A $\beta$ 1-7-C
3	DAEFRHDSG-C	A $\beta$ 1-9-C
4	DAEFRHDSGYEV-C	A $\beta$ 1-12-C
5	DAEFR-GAGA-C	A $\beta$ 1-5-L-C
6	DAEFRHD-GAGA-C	A $\beta$ 1-7-L-C
7	DAEFRHDSG-GAGA-C	A $\beta$ 1-9-L-C
8	DAEFRHDSGYEV-GAGA-C	A $\beta$ 1-12-L-C
9	VEYGSDHRFEAD-C	A $\beta$ 12-1-C
10	GAGA	Linker peptide
11	PKYVKQNTLKLAT	Influenza Hemagglutinin: HA <sub>307-319</sub>
12	AKXVAAWTLKAAA	PADRE
13	EKKIAKMEKASSVFNV	Malaria CS: T3 epitope
14	FELLTRILTI	Hepatitis B surface antigen: HB <sub>e</sub> Ag <sub>19-28</sub>
15	DQSIGDLIAEAMDVKVGNEG	Heat Shock Protein 65: hsp65 <sub>153-171</sub>
16	QVHFQPLPPAVVKL	Bacillus Calmette-Guerin (BCG)
17	QYIKANSKFIGITEL	Tetanus toxoid: TT <sub>830-844</sub>
18	FNNFTVSFWLRLVPKVSASHLE	Tetanus toxoid: TT <sub>947-967</sub>
19	KQIIINMWQEVGKAMY	HIV gp120 T1
20	DAEFRHD-QYIKANSKFIGITEL-C-FNNFTVSFWLRLVPKVSASHLE-DAEFRHD	A $\beta$ <sub>1-7</sub> /TT <sub>830-844</sub> /C/TT <sub>947-967</sub> /A $\beta$ <sub>1-7</sub>
21	DAEFRHDSGYEVHHQQLVFFAEDVGSNK GAIIGLMVGGVIA	A $\beta$ <sub>1-42</sub>

22	DAEFRHDQYIKANSKFIGITEL	AN90549: A $\beta_{1-7}$ /TT <sub>830-844</sub> (used in a MAP4 configuration)
23	DAEFRHDFNNFTVSFWLRVPKVSASHLE	AN90550: A $\beta_{1-7}$ /TT <sub>947-967</sub> (used in a MAP4 configuration)
24	DAEFRHD-QYIKANSKFIGITELFNNFTVSFWLRVPKVSASHLE	AN90542: A $\beta_{1-7}$ /TT <sub>830-844</sub> + TT <sub>947-967</sub> (used in a linear configuration)
25	EFRHDSG-QYIKANSKFIGITEL	AN90576: A $\beta_{3-9}$ /TT <sub>830-844</sub> (used in a MAP4 configuration)
26	AKXVAAWTLKAAA-DAEFRHD	AN90562: A $\beta_{1-7}$ /PADRE
27	DAEFRHD-DAEFRHDD-AEFRHDAKXVAAWTLKAAA	AN90543: A $\beta_{1-7}$ x 3/PADRE
28	AKXVAAWTLKAAA-DAEFRHD-DAEFRHD-DAEFRHD	PADRE/A $\beta_{1-7}$ x 3
29	DAEFRHD-AKXVAAWTLKAAA	A $\beta_{1-7}$ x 3/PADRE
30	DAEFRHD-ISQAVHAAHAEINEAGR	A $\beta_{1-7}$ /albumin fragment
31	FRHDSGY-ISQAVHAAHAEINEAGR	A $\beta_{4-10}$ / albumin fragment
32	EFRHDSG-ISQAVHAAHAEINEAGR	A $\beta_{3-9}$ / albumin fragment
33	PKYVKQNTLKLAT-DAEFRHD-DAEFRHD-DAEFRHD	HA <sub>307-319</sub> /A $\beta_{1-7}$ x 3
34	DAEFRHD-PKYVKQNTLKLAT-DAEFRHD	A $\beta_{1-7}$ /HA <sub>307-319</sub> /A $\beta_{1-7}$
35	DAEFRHD-DAEFRHD-DAEFRHD-PKYVKQNTLKLAT	A $\beta_{1-7}$ x 3/ HA <sub>307-319</sub>
36	DAEFRHD-DAEFRHD-PKYVKQNTLKLAT	A $\beta_{1-7}$ x 2/ HA <sub>307-319</sub>
37	DAEFRHD-PKYVKQNTLKLAT-EKKIAKMEKASSVFNV-QYIKANSKFIGITEL-FNNFTVSFWLRVPKVSASHLE-DAEFRHD	A $\beta_{1-7}$ /HA <sub>307-319</sub> /Malaria CS/ TT <sub>830-844</sub> /TT <sub>947-967</sub> /A $\beta_{1-7}$
38	DAEFRHD-DAEFRHD-DAEFRHD-QYIKANSKFIGITEL-C-FNNFTVSFWLRVPKVSASHLE	A $\beta_{1-7}$ x 3/TT <sub>830-844</sub> /C/TT <sub>947-967</sub>
39	DAEFRHD-QYIKANSKFIGITEL-C-FNNFTVSFWLRVPKVSASHLE	A $\beta_{1-7}$ /TT <sub>830-844</sub> /C/TT <sub>947-967</sub>

40	GADDVV/DSSKSFVMENFSSYHGTKPGY VDSIQKGIQKPKSGTQGNYDDDWWKEFYS TDNKYDAAGYSVDNENPLSGKAGGVVK VTPYGLTKVLALKVDNAETIKKELGLSLTE PLMEQVGTEEFIKRFGDGAWSRVVLSLPFA EGSSSVEYINNNWEQAKALSVELEINFETR GKRGQDAMYEMQAQACAGNRVRRSVG SSLSCINLDWDVIRDKTKIESLKEHGPI KNKMSESPNKTVSEEKAKQYLEEHQTA LEHPELSELKTVGTGTPVAGANYAAWA VNVAQVIDSETADNLEKTTAALSILPGIGS VMGIADGAVHHNTEEIVAQSIALLSMVA QAIPLVGELVDIGFAAYNFVESIINLFQVV HNSYNRPAVSPGHKTQPFLHDGYAVSW NTVEDSIIRTGFQGESGHDIKITAENTPLPI AGVLLPTIPGKLDVNKSHTHISVNGRKIRM RCRAIDGVTFCRPKSPVYVGNGVHANL HVAFHRSSEKIHNSNEISSDSIGVLGYQK TVDHTKVNSKLSLFFEIKS	CRM $\beta$ <sub>107</sub>
41	ISQAVHAAHAEINEAGR	Albumin fragment

#### DETAILED DESCRIPTION OF THE INVENTION

5 The present invention is directed to methods of generating peptide immunogen-carrier conjugates wherein the unreacted active functional groups on the carrier which are generated during activation are inactivated by using capping reagents such as *N*-Acetyl cysteamine in order to prevent them from reacting further. The present invention is also directed to capped carrier-peptide immunogen conjugates generated  
10 by those methods and to immunogenic compositions comprising said conjugates.

The approach of increasing immunogenicity of small or poorly immunogenic molecules, such as saccharides, through conjugation has been utilized successfully for decades (see, e.g., Goebel *et al.* (1939) *J. Exp. Med.* 69: 53), and many  
15 immunogenic compositions have been described in which purified capsular polymers have been conjugated to carrier proteins to create more effective immunogenic compositions by exploiting this "carrier effect". For example, Schneerson *et al.* (*J. Exp. Med.* 152: 361-376, 1980), describe *Haemophilus influenzae* b polysaccharide

protein conjugates that confer immunity to invasive diseases caused by that microorganism. Conjugates of PRP (polyribosyribitol phosphate, a capsular polymer of *H. influenzae* b) have been shown to be more effective than immunogenic compositions based on the polysaccharide alone (Chu *et al.*, (1983) *Infect. Immun.* 40: 245; Schneerson *et al.* (1984), *Infect. Immun.* 45: 582-591). Conjugation has also been shown to bypass the poor antibody response usually observed in infants when immunized with a free polysaccharide (Anderson *et al.* (1985) *J. Pediatr.* 107: 346; Insel *et al.* (1986) *J. Exp. Med.* 158: 294).

5 10 A further advantage of using as the protein carrier a bacterial toxin or toxoid against which routine immunization of humans (e.g., tetanus or diphtheria) is a standard practice is that a desired immunity to the toxin or toxoid is induced along with immunity against the pathogens associated with the capsular polymer.

15 20 25 30 15 Antigenic determinant/hapten-carrier conjugates also are being used to produce highly specific monoclonal antibodies that can recognize discrete chemical epitopes on the coupled hapten. The resulting monoclonals often are used to investigate the epitopic structure and interactions between native proteins. In many cases, the antigenic determinants/haptens used to generate these monoclonals are small peptide segments representing crucial antigenic sites on the surface of larger proteins. The criteria for a successful carrier to be used in generating an antigenic determinant/hapten-carrier conjugate are the potential for immunogenicity, the presence of suitable functional groups for conjugation with an antigenic determinant/hapten, reasonable solubility properties even after derivatization and lack of toxicity *in vivo*.

These criteria are met by the conjugates generated by the methods of the instant invention. The conjugates may be any stable peptide immunogen-carrier conjugates generated using the conjugation process described herein. The conjugates are generated using a process of the instant invention wherein a protein/polypeptide carrier having the following structure:

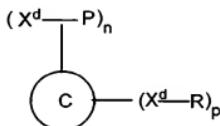


is covalently attached to a protein/polypeptide carrier,  
wherein,

C is a protein/polypeptide carrier and X is a derivatizable functional group on

5 an amino acid residue on the protein/polypeptide carrier or optionally on an amino acid residue on a peptide linker covalently attached to the protein/polypeptide carrier, and wherein m is an integer greater than 0, but less than or equal to 85, is covalently attached to a peptide immunogen and wherein the peptide immunogen-protein/polypeptide carrier conjugate has the following formula, is represented by the

10 following formula:



wherein,

C is the protein/polypeptide carrier and X<sup>d</sup> is a derivatized functional group on

15 an amino acid residue on the protein/polypeptide carrier or optionally on an amino acid residue on a peptide linker covalently attached to the protein/polypeptide carrier, P is a peptide immunogen covalently attached to the derivatized functional group on the amino acid residue on the protein/polypeptide carrier or optionally on an amino acid residue on a peptide linker covalently attached to a protein/polypeptide carrier, R is a capping molecule covalently attached to the derivatized functional group on an amino acid residue on the protein/polypeptide carrier or optionally on an amino acid residue on a peptide linker covalently attached to a protein/polypeptide carrier thereby preserving the functionality of the carrier, such that it retains its ability to elicit the desired immune responses against the peptide immunogen that would otherwise

20

not occur without a carrier., n is an integer greater than 0, but less than or equal to 85, and p is an integer greater than 0, but less than 85.

#### A. Selection Of Carriers

Some peptide immunogens contain the appropriate epitope for inducing an immune response, but are too small to be immunogenic. In this situation, the peptide immunogens are linked to a suitable carrier to help elicit an immune response. In the above schematic representation of the peptide immunogens-carrier conjugate generated by a process of the present invention, C is a protein/polypeptide carrier to which peptide immunogens are conjugated directly via derivatized functional groups on amino acid residues on the carrier themselves or indirectly via derivatized functional groups on peptide linkers covalently attached to the carriers. Suitable protein/polypeptide carriers include, but are not limited to, albumin (including human serum albumin), keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, tetanus toxoid, or a toxoid from other pathogenic bacteria having reduced toxicity, including mutants, such as diphtheria, *E. coli*, cholera, or *H. pylori*, or an attenuated toxin derivative. One such carrier is the CRM<sub>197</sub> protein (SEQ ID NO.:40) that is cross-reactive with diphtheria toxin.

Other carriers include T-cell epitopes that bind to multiple MHC alleles, e.g., at least 75% of all human MHC alleles. Such carriers are sometimes known in the art as "universal T-cell epitopes." Exemplary carriers with universal T-cell epitopes include:

Influenza Hemagglutinin: HA <sub>307-319</sub>	PKYVKQNTLKLAT (SEQ. ID NO. 11)
PAN-DR Peptide (PADRE peptide)	AKXVAATWLKAAA (SEQ. ID NO. 12)
Malaria CS: T3 epitope	EKKIAKMEKASSVFNV (SEQ. ID NO. 13)
Hepatitis B surface antigen: HB <sub>s</sub> Ag <sub>19-28</sub>	FELLTRILTI (SEQ. ID NO. 14)
Heat Shock Protein 65: hsp65 <sub>153-171</sub>	QSIGDLIAEAMDVKVGNEG (SEQ. ID NO. 15)
Bacillus Calmette-Guerin (BCG)	QVHFQPLPPAVVKL(SEQ. ID NO. 16)
Tetanus toxoid: TT <sub>830-844</sub>	QYIKANSKFIGITEL (SEQ. ID NO.

17)

Tetanus toxoid: TT<sub>947-967</sub>

NNFTVSFWLRVPKVSASHLE

(SEQ. ID NO. 18)

HIV gp120 T1:

KQIINMWQEVGKAMY (SEQ. ID NO. 19)

CRM<sub>197</sub>See the Brief Description of the Sequences section  
(SEQ ID NO.:40)

Albumin fragment

ISQAVHAAHAEINEAGR (SEQ ID NO: 41)

Other carriers for stimulating or enhancing an immune response and to which a peptide immunogen or a hapten can be conjugated include cytokines such as IL-1, IL-1  $\alpha$  and  $\beta$  peptides, IL-2,  $\gamma$ INF, IL-10, GM-CSF, and chemokines, such as MIP 1 $\alpha$

5 and  $\beta$  and RANTES. Immunogenic peptides can also be linked to proteins/peptide carriers that enhance transport across tissues, as described in O'Mahony, WO 97/17163 and WO 97/17614, which are hereby incorporated by reference.

Still further carriers include recombinant Streptococcal C5a peptidase, *Streptococcus 10 pyogenes* ORFs 1224, 1664 and 2452, *Chlamydia pneumoniae* ORFs T367 and T858, growth factors, and hormones.

In one preferred embodiment of the present invention, the carrier protein is CRM<sub>197</sub>, a non-toxic mutant of diphtheria toxin with one amino acid change in its primary 15 sequence. The glycine present at the amino acid position 52 of the molecule is replaced with a glutamic acid due to a single nucleic acid codon change. Due to this change, the protein lacks ADP-ribosyl transferase activity and becomes non-toxic. It has a molecular weight of 58,408 Da. CRM<sub>197</sub> is produced in large quantities by recombinant expression in accordance with U.S. Patent 5,614,382, which is hereby 20 incorporated by reference. Conjugations of saccharides as well as peptides to CRM<sub>197</sub> are carried out by linking through the  $\epsilon$ -amino groups of lysine residues. It has been well established through several commercial products that CRM<sub>197</sub> is an excellent and safe carrier for B-cell epitopes.

**B. Immunogenic Peptides**

As used herein, the term "*peptide immunogen*" or "*haptens*" is any protein or subunit structure/fragment/analog derived therefrom that can elicit, facilitate, or be induced to produce an immune response on administration to a mammal. In particular, the term

5 is used to refer to a polypeptide antigenic determinant from any source (bacteria, virus or eukaryote), which may be coupled to a carrier using a method disclosed herein. Such polypeptide immunogen/antigenic determinants may be of viral, bacterial or eukaryotic cell origin.

10 Peptide immunogens from a bacterial cell include those derived from bacterial cell surface or secreted proteins, which can be used in protein-based immunogenic compositions. Exemplary bacterial strains include *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Neisseria meningitidis*, *Haemophilus influenzae*, *Klebsiella* spp., *Pseudomonas* spp., *Salmonella* spp., *Shigella* spp., *Alloiococcus otiditis*, and

15 15 Group B *streptococci*.

Exemplary peptide immunogens from viruses include those derived from human immunodeficiency virus (HIV), herpes simplex virus (HSV), human papilloma virus (HPV), parainfluenza virus (PIV), vesicular stomatitis virus (VSV), respiratory

20 syncytial virus (RSV), Epstein-Barr virus (EBV), coronavirus, vaccinia virus, rotavirus, rabies virus, hepatitis C virus (HCV) and hepatitis B virus (HBV) to name a few.

Exemplary fungal peptide immunogens include those derived from *Candida albicans*,

25 25 *Cryptococcus neoformans*, *Coccidioides* spp., *Histoplasma* spp., and *Aspergillus* spp. Parasitic antigens include those derived from *Plasmodium* spp., *Trypanosoma* spp., *Schistosoma* spp., *Leishmania* spp. and the like.

Exemplary eukaryotic peptide immunogens that can be conjugated to a carrier for

30 use as an immunotherapeutic in the prevention, treatment, prophylaxis or amelioration of various human diseases include those associated with tumor cells, those derived from A $\beta$ , a peptide of 39-43 amino acids, preferably 42 amino acids, which is the principal component of characteristic plaques of Alzheimer's disease (AD) (see US 4,666,829; Glenner & Wong (1984) *Biochem. Biophys. Res. Commun.*

120: 1131, Hardy (1984) *TINS* 20: 1131; Hardy (1977) *TINS* 20: 154), those derived from amyloid peptides of amylin, a polypeptide material produced by pancreatic islet cells that has been implicated in type II diabetes, peptides derived from low density lipoprotein gene products, which have been implicated in 5 atherosclerosis and antigenic peptides derived from inflammatory cytokines and growth factors such as interleukin 6 (IL-6), tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and GDF-8. Such eukaryotic peptide immunogens may include either T-cell (CTL) or B-cell epitope.

10 A "CTL epitope" is one derived from selected epitopic regions of potential target antigens, such as tumor associated antigens, including, but not limited to, renal cell carcinoma, breast cancer, carcinoembryonic antigens, melanoma (MAGE) antigens, and prostate cancer specific antigens such as prostate specific membrane antigen (PSMA) and prostate stem cell antigen (PSCA), hepatitis C antigens,

15 A $\beta$ , also known as  $\beta$ -amyloid peptide, or A4 peptide (see US 4,666,829; Glenner & Wong, *Biochem. Biophys. Res. Commun.*, 120, 1131 (1984)), is a peptide of 39-43 amino acids, which is the principal component of characteristic plaques of Alzheimer's disease. A $\beta$  is generated by processing of a larger protein APP by two 20 enzymes, termed  $\beta$  and  $\gamma$  secretases (see Hardy, *TINS* 20, 154 (1997)). Known mutations in APP associated with Alzheimer's disease occur proximate to the site of  $\beta$  or  $\gamma$  secretase, or within A $\beta$ . For example, position 717 is proximate to the site of  $\gamma$ -secretase cleavage of APP in its processing to A $\beta$ , and positions 670/671 are proximate to the site of  $\beta$ -secretase cleavage. It is believed that the mutations cause 25 AD by interacting with the cleavage reactions by which A $\beta$  is formed so as to increase the amount of the 42/43 amino acid form of A $\beta$  generated.

A $\beta$  has the unusual property that it can fix and activate both classical and alternate complement cascades. In particular, it binds to C1q and ultimately to C3bi. This 30 association facilitates binding to macrophages leading to activation of B cells. In addition, C3bi breaks down further and then binds to CR2 on B cells in a T cell dependent manner leading to a 10,000-fold increase in activation of these cells. This

mechanism causes A $\beta$  to generate an immune response in excess of that of other antigens.

A $\beta$  has several natural occurring forms. The human forms of A $\beta$  are referred to as

5 A $\beta$ 39, A $\beta$ 40, A $\beta$ 41, A $\beta$ 42 and A $\beta$ 43. The sequences of these peptides and their relationship to the APP precursor are illustrated by Fig. 1 of Hardy et al., TINS 20, 155-158 (1997). For example, A $\beta$ 42 has the sequence:

H<sub>2</sub>N-Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-  
His-His-Gln-Lys-Leu-Val-Phe-Ala-Glu-Asp-Val-Gly-  
10 Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-  
Val-Val-Ile-Ala-OH (SEQ ID NO. 21).

A $\beta$ 41, A $\beta$ 40 and A $\beta$ 39 differ from A $\beta$ 42 by the omission of Ala, Ala-Ile, and Ala-Ile-Val respectively from the C-terminal end. A $\beta$ 43 differs from A $\beta$ 42 by the presence of a threonine residue at the C-terminus.

15

Peptide immunogens which are fragments of A $\beta$  are advantageous relative to the intact molecule for use in the present methods for several reasons. First, because only certain epitopes within A $\beta$  induce a useful immunogenic response for treatment of Alzheimer's disease, an equal dosage of mass of a fragment containing such

20 epitopes provides a greater molar concentration of the useful immunogenic epitopes than a dosage of intact A $\beta$ . Second, certain peptide immunogens of A $\beta$  generate an immunogenic response against amyloid deposits without generating a significant immunogenic response against APP protein from which A $\beta$  derives. Third, peptide immunogens of A $\beta$  are simpler to manufacture than intact A $\beta$  due to their shorter  
25 size. Fourth, peptide immunogens of A $\beta$  do not aggregate in the same manner as intact A $\beta$ , simplifying preparation of conjugates with carriers.

Some peptide immunogens of A $\beta$  have a sequence of at least 2, 3, 5, 6, 10, or 20 contiguous amino acids from a natural peptide. Some peptide immunogens have no more than 10, 9, 8, 7, 5 or 3 contiguous residues from A $\beta$ . In a preferred

30 embodiment, peptide immunogens from the N-terminal half of A $\beta$  are used for preparing conjugates. Preferred peptide immunogens include A $\beta$ 1-5, 1-6, 1-7, 1-10, 1-11, 3-7, 1-3, and 1-4. The designation A $\beta$ 1-5 for example, indicates an N-terminal

fragment including residues 1-5 of A $\beta$ . A $\beta$  fragments beginning at the N-terminus and ending at a residue within residues 7-11 of A $\beta$  are particularly preferred. The fragment A $\beta$ 1-12 can also be used but is less preferred. In some methods, the fragment is an N-terminal fragment other than A $\beta$ 1-10. Other preferred fragments

5 include A $\beta$ 13-28, 15-24, 17-28, 1-28, 25-35, 35-40, 35-42 and other internal fragments and C-terminus fragments. These fragments require screening for activity in clearing or preventing amyloid deposits (see WO 00/72880, which is incorporated herein in its entirety for all purposes). Administration of N-terminal fragments of A $\beta$  induces the production of antibodies that recognize A $\beta$  deposits *in vivo* and *in vitro*.

10 Fragments lacking at least one, and sometimes at least 5 or 10 C-terminal amino acids present in naturally occurring forms of A $\beta$  are used in some methods. For example, a fragment lacking 5 amino acids from the C-terminal end of A $\beta$ 43 includes the first 38 amino acids from the N-terminal end of A $\beta$ .

Unless otherwise indicated, reference to A $\beta$  includes the natural human amino acid sequences indicated above as well as analogs including allelic, species and induced variants. Analogs typically differ from naturally occurring peptides at one, two or a few positions, often by virtue of conservative substitutions. Analogs typically exhibit at least 80 or 90% sequence identity with natural peptides. Some analogs also include unnatural amino acids or modifications of N- or C-terminal amino acids at one, two, or a few positions. For example, the natural aspartic acid residue at position 1 and/or 7 of A $\beta$  can be replaced with iso-aspartic acid.

Examples of unnatural amino acids are D, alpha, alpha-disubstituted amino acids, N-alkyl amino acids, lactic acid, 4-hydroxyproline, gamma-carboxyglutamate, epsilon-N,N,N-trimethyllysine, epsilon-N-acetyllysine, O-phosphoserine, N-acetylsine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, omega-N-methylarginine,  $\beta$ -alanine, ornithine, norleucine, norvaline, hydroxyproline, thyroxine, gamma-amino butyric acid, homoserine, citrulline, and isoaspartic acid. Immunogenic peptides also include analogs of A $\beta$  and fragments thereof. Some therapeutic agents of the invention are all-D peptides, e.g., all-D A $\beta$ , all-D A $\beta$  fragment, or analogs of all-D A $\beta$

25 or all-D A $\beta$  fragment. Fragments and analogs can be screened for prophylactic or

30

therapeutic efficacy in transgenic animal models in comparison with untreated or placebo controls as described in WO 00/72880.

Peptide immunogens also include longer polypeptides that include, for example, an immunogenic of A $\beta$  peptide, together with other amino acids. For example, preferred

5 immunogenic peptides include fusion proteins comprising a segment of A $\beta$  linked to a heterologous amino acid sequence that induces a helper T-cell response against the heterologous amino acid sequence and thereby a B-cell response against the A $\beta$  segment. Such polypeptides can be screened for prophylactic or therapeutic efficacy in animal models in comparison with untreated or placebo controls as described in

10 WO 00/72880.

The A $\beta$  peptide, analog, immunogenic fragment or other polypeptide can be administered in disaggregated or aggregated form. Disaggregated A $\beta$  or fragments thereof means monomeric peptide units. Disaggregated A $\beta$  or fragments thereof are generally soluble, and are capable of self-aggregating to form soluble oligomers.

15 Oligomers of A $\beta$  and fragments thereof are usually soluble and exist predominantly as alpha-helices or random coils. Aggregated A $\beta$  or fragments thereof means oligomers of A $\beta$  or fragments thereof that have associate into insoluble beta-sheet assemblies. Aggregated A $\beta$  or fragments thereof also means fibrillar polymers. Fibrils are usually insoluble. Some antibodies bind either soluble A $\beta$  or fragments

20 thereof or aggregated A $\beta$  or fragments thereof. Some antibodies bind both soluble A $\beta$  or fragments thereof and aggregated A $\beta$  or fragments thereof.

Immunogenic peptides also include multimers of monomeric immunogenic peptides. Immunogenic peptides other than A $\beta$  peptides should induce an immunogenic response against one or more of the preferred fragments of A $\beta$  listed above (e.g.,

25 A $\beta$ 1-3, 1-7, 1-10, and 3-7).

Immunogenic peptides of the present invention are linked to a carrier using a method of the present invention to form a conjugate. The immunogenic peptide can be linked at its amino terminus, its carboxyl terminus, or both to a carrier to form a conjugate. Optionally, multiple repeats of the immunogenic peptide can be present in the

30 conjugate.

An N-terminal fragment of A $\beta$  can be linked at its C-terminus to a carrier peptide to form a conjugate. In such conjugates, the N-terminal residue of the fragment of A $\beta$  constitutes the N-terminal residue of the conjugate. Accordingly, such conjugates are effective in inducing antibodies that bind to an epitope that requires the N-  
5 terminal residue of A $\beta$  to be in free form. Some immunogenic peptides of the invention comprise a plurality of repeats of an N-terminal segment of A $\beta$  linked at the C-terminus to one or more copy of a carrier peptide to form a conjugate. The N-terminal fragment of A $\beta$  incorporated into such conjugates sometimes begins at A $\beta$ 1-3 and ends at A $\beta$ 7-11. A $\beta$ 1-7, 1-3, 1-4, 1-5, and 3-7 are preferred N-terminal  
10 fragment of A $\beta$ . Some conjugates comprise different N-terminal segments of A $\beta$  in tandem. For example, a conjugate can comprise A $\beta$ 1-7 followed by A $\beta$ 1-3 linked to a carrier.

In some conjugates, an N-terminal segment of A $\beta$  is linked at its N-terminal end to a  
15 carrier peptide. The same variety of N-terminal segments of A $\beta$  can be used as with C-terminal linkage. Some conjugates comprise a carrier peptide linked to the N-terminus of an N-terminal segment of A $\beta$ , which is in turn linked to one or more additional N-terminal segments of A $\beta$  in tandem. Preferably, such immunogenic A $\beta$  fragments, once conjugated to an appropriate carrier, induce an immunogenic  
20 response that is specifically directed to the A $\beta$  fragment without being directed to other fragments of A $\beta$ .

Immunogenic peptides of the invention include immunogenic heterologous peptides. In some immunogenic peptides, an A $\beta$  fragment is linked to a carrier to form an immunogenic heterologous peptide. This heterologous peptide is linked to a carrier  
25 using a method of the present invention to form a conjugate. Some of these immunogenic heterologous peptides comprise fragments of A $\beta$  linked to tetanus toxoid epitopes such as described in US 5,196,512, EP 378,881 and EP 427,347. Optionally, an immunogenic peptide can be linked to one or multiple copies of a carrier, for example, at both the N and C termini of the carrier to form an  
30 immunogenic heterologous peptide. Other of these immunogenic heterologous peptides comprise fragments of A $\beta$  linked to carrier peptides described in US 5,736,142. For example, an immunogenic heterologous peptide can comprise A $\beta$ 1-7

followed by A $\beta$ 1-3 followed by a carrier. Examples of such immunogenic heterologous peptides include:

A $\beta$  1-7/Tetanus toxoid 830-844 + 947-967 in a linear configuration

DAEFRHD-QYIKANSKFIGITELFNNFTVSFWLRVPKVSASHLE (SEQ ID NO.:24)

5 NO.:24)

Peptides described in US 5,736,142 (all in linear configurations):

PADRE/A $\beta$  1-7:

AKXVAAWTLKAAA-DAEFRHD (SEQ ID NO.:26)

A $\beta$ 1-7 x 3/PADRE:

10 DAEFRHD-DAEFRHD-DAEFRHD-AKXVAAWTLKAAA (SEQ ID NO.:27)

PADRE/A $\beta$ 1-7 x 3:

AKXVAAWTLKAAA-DAEFRHD-DAEFRHD-DAEFRHD (SEQ ID NO.:28)

A $\beta$ 1-7/PADRE:

DAEFRHD-AKXVAAWTLKAAA (SEQ ID NO.:29)

15 A $\beta$ 1-7/albumin fragment:

DAEFRHD-ISQAVHAAHAEINEAGR (SEQ ID NO.:30)

A $\beta$ 4-10/albumin fragment:

FRHDSGY-ISQAVHAAHAEINEAGR (SEQ ID NO.:31)

A $\beta$ 3-9/albumin fragment:

20 EFRHDSG-ISQAVHAAHAEINEAGR (SEQ ID NO.:32)

HA<sub>307-319</sub>/A $\beta$ 1-7 x 3:

PKYVKQNTLKLAT-DAEFRHD-DAEFRHD-DAEFRHD (SEQ ID NO.:33)

A $\beta$ 1-7/HA<sub>307-319</sub>/A $\beta$ 1-7:

DAEFRHD-PKYVKQNTLKLAT-DAEFRHD (SEQ ID NO.:34)

25 A $\beta$ 1-7 x 3/HA<sub>307-319</sub>:

DAEFRHD-DAEFRHD-DAEFRHD-PKYVKQNTLKLAT (SEQ ID NO.:35)

A $\beta$ 1-7 x 2/HA<sub>307-319</sub>:

DAEFRHD-DAEFRHD-PKYVKQNTLKLAT (SEQ ID NO.:36)

A $\beta$ 1-7/HA<sub>307-319</sub>/Malaria CS/TT<sub>830-844</sub>/TT<sub>947-967</sub>/A $\beta$ 1-7

30 DAEFRHD-PKYVKQNTLKLAT-EKKIAKMEKASSVFNV-QYIKANSKFIGITEL-

FNNFTVSFWLRVPKVSASHLE-DAEFRHD (SEQ ID NO.:37)

A $\beta$ 1-7 x 3/TT<sub>830-844</sub>/C/TT<sub>947-967</sub>

DAEFRHD-DAEFRHD-DAEFRHD-QYIKANSKFIGITEL-C-  
FNNFTVSFWLRVPKVSASHLE (SEQ ID NO.:38)

$\text{A}\beta_{1.7}/\text{TT}_{830-844}/\text{C}/\text{TT}_{947-957}$

5 DAEFRHD-YIKANSKFIGITELCFNNFTVSFWLRVPKVSASHLE (SEQ ID  
NO.:39)

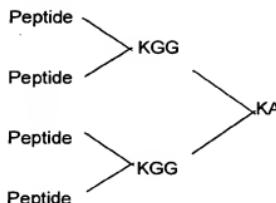
$\text{A}\beta_{1.7}/\text{TT}_{830-844}/\text{C}/\text{TT}_{947-957}/\text{A}\beta_{1.7}$

DAEFRHD-QYIKANSKFIGITEL-C-FNNFTVSFWLRVPKVSASHLE-  
DAEFRHD (SEQ ID NO.:20)

Some immunogenic heterologous peptides comprise a multimer of immunogenic peptides represented by the formula  $2^x$ , in which x is an integer from 1-5. Preferably x is 1, 2 or 3, with 2 being most preferred. When x is two, such a multimer has four immunogenic peptides linked in a preferred configuration referred to as MAP4 (see US 5,229,490). Such immunogenic peptides are then linked to a carrier using a method of the present invention to form a conjugate.

10 15 The MAP4 configuration is shown below, where branched structures are produced by initiating peptide synthesis at both the N-terminal and side chain amines of lysine. Depending upon the number of times lysine is incorporated into the sequence and allowed to branch, the resulting structure will present multiple N-termini. In this example, four identical N-termini have been produced on the branched lysine-  
20 containing core. Such multiplicity greatly enhances the responsiveness of cognate B cells.

25



30 Examples of such immunogenic heterologous peptides include:

$\text{A}\beta 1.7/\text{Tetanus toxoid 830-844}$  in a MAP4 configuration:

DAEFRHD-QYIKANSKFIGITEL (SEQ ID NO.:22)

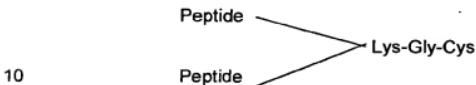
A $\beta$  1-7/Tetanus toxoid 947-967 in a MAP4 configuration:

DAEFRHD-FNNFTVSFWLRVPKVSASHLE (SEQ ID NO.:23)

A $\beta$  3-9/Tetanus toxoid 830-844 in a MAP4 configuration:

5 EFRHDSG-QYIKANSKFIGITEL (SEQ ID NO.:25)

DAEFRHD-QYIKANSKFIGITEL on a 2 branched resin



The A $\beta$  peptide, analog, active fragment or other polypeptide can be administered in associated or multimeric form or in dissociated form. Therapeutic agents also include multimers of monomeric immunogenic agents. Agents other than 15 A $\beta$  peptides should induce an immunogenic response against one or more of the preferred fragments of A $\beta$  listed above (e.g., 1-10, 1-7, 1-3, and 3-7), and can also be conjugated to a carrier using a method of the present invention. Preferably, such agents, once conjugated to an appropriate carrier, induce an immunogenic response that is specifically directed to one of these fragments without being directed to other 20 fragments of A $\beta$ . To facilitate the conjugation of an peptide immunogen with a carrier, additional amino acids can be added to the termini of the antigenic determinants. The additional residues can also be used for modifying the physical or chemical properties of the peptide immunogen. Amino acids such as tyrosine, cysteine, lysine, glutamic or aspartic acid, or the like, can be introduced at the C- or 25 N- terminus of the peptide immunogen. Additionally, peptide linkers containing amino acids such as glycine and alanine can also be introduced. In addition, the antigenic determinants can differ from the natural sequence by being modified by terminal NH<sub>2</sub>-group acylation, e.g., by alkanoyl (C1-C20) or thioglycolyl acetylation, terminal-carboxy amidation, e.g., ammonia, methylamine, etc. In some instances these 30 modifications may provide sites for linking to a support or other molecule.

The peptide immunogens used to generate conjugates of the present invention using a process disclosed herein can be combined via linkage to form polymers (multimers), or can be formulated in a composition without linkage, as an admixture. Where a peptide is linked to an identical peptide, thereby forming a homopolymer, a 5 plurality of repeating epitopic units are presented. For example, multiple antigen peptide (MAP) technology is used to construct polymers containing both CTL and/or antibody peptides and peptides. When the peptides differ, e.g., a cocktail representing different viral subtypes, different epitopes within a subtype, different HLA restriction specificities, or peptides which contain T-helper epitopes, 10 heteropolymers with repeating units are provided. In addition to covalent linkages, noncovalent linkages capable of forming intermolecular and intrastructural bonds are also contemplated.

Such peptide immunogens and their analogs are synthesized by solid phase peptide 15 synthesis or recombinant expression, or are obtained from natural sources. Automatic peptide synthesizers are commercially available from numerous suppliers, such as Applied Biosystems, Foster City, California.

Recombinant expression can be in bacteria (such as *E. coli*), yeast, insect cells or 20 mammalian cells. Procedures for recombinant expression are described by Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Press, NY, 2nd ed., 1989). Some immunogenic peptides are also available commercially (e.g., American Peptides Company, Inc., Sunnyvale, CA, and California Peptide Research, Inc., Napa, CA).

25 Random libraries of peptides or other compounds can also be screened for suitability as a peptide immunogen. Combinatorial libraries can be produced for many types of compounds that can be synthesized in a step-by-step fashion. Such compounds include polypeptides, beta-turn mimetics, hormones, oligomeric *N*-substituted 30 glycines, and oligocarbamates and the like. Large combinatorial libraries of the compounds can be constructed by the encoded synthetic libraries (ESL) method described in WO 95/12608, WO 93/06121, WO 94/08051, WO 95/35503 and WO 95/30642 (each of which is incorporated by reference for all purposes). Peptide

libraries can also be generated by phage display methods (see, e.g., Devlin, WO 91/18980).

**C. Derivatization and Conjugation of An Immunogenic Peptide to a Protein Carrier**

5 The site of attachment of a peptide immunogen to a protein/polypeptide carrier, and the nature of the cross-linking agent that is used to attach a peptide immunogen to the carrier are both important to the specificity of the resultant antibody generated against it. For proper recognition, the peptide immunogen must be coupled to the carrier with the appropriate orientation. For an antibody to recognize subsequently 10 the free peptide immunogens without carrier , the peptide immunogen-protein/polypeptide carrier conjugate must present the peptide immunogens in an exposed and accessible form. Optimal orientation is often achieved by directing the cross-linking reaction to specific sites on the peptide immunogens. One way to achieve this with a peptide immunogen is by attaching a terminal cysteine residue 15 during peptide synthesis. This provides a sulfhydryl group on one end of the peptide for conjugation to the carrier. Cross-linking through this group provides attachment of the peptide immunogen only at one end, thereby ensuring consistent orientation.

20 In peptide immunogen-carrier conjugation, the goal is not to maintain the native state or stability of the carrier, but to present the hapten in the best possible way to the immune system. In reaching this goal, the choice of conjugation chemistry may control the resultant titer, affinity, and specificity of the antibodies generated against the hapten. It may be important in some cases to choose a cross-linking agent 25 containing a spacer arm long enough to present the antigen in an unrestricted fashion. It also may be important to control the density of the peptide immunogen on the surface of the carrier. Too little peptide immunogen substitution may result in little or no response. A peptide immunogen density too high actually may cause immunological suppression and decrease the response. In addition, the cross-linker itself may generate an undesired immune response. These issues need to be taken 30 into consideration in selecting not only the appropriate cross-linking reagents, but also the appropriate ratios of protein/polypeptide carrier and peptide immunogen.

A variety of means of attaching the protein/peptide carriers to the peptide immunogens are possible. Ionic interactions are possible through the termini or through the  $\epsilon$ -amino group of lysine. Hydrogen bonding between the side groups of the residues and the peptide immunogen are also possible. Finally, conformation interactions between the protein/peptide carriers and the immunogenic peptide may give rise to a stable attachment.

Peptide immunogens-carrier conjugates have been successfully generated using various cross-linking reagents such as zero-length, homobifunctional or heterobifunctional cross linkers. The smallest available reagent systems for bioconjugation are the so-called zero-length cross-linkers. These compounds mediate the conjugation of two molecules by forming a bond containing no additional atoms. Thus, one atom of a molecule is spacer. In many conjugation schemes, the final complex is bound together by virtue of chemical components that add foreign structures to the substances being cross-linked. In some applications, the presence of these intervening linkers may be detrimental to the intended use. For instance, in the preparation of peptide immunogen-carrier conjugates the complex is formed with the intention of generating an immune response to the attached hapten. Occasionally, a portion of the antibodies produced by this response will have specificity for the cross-linking agent used in the conjugation procedure. Zero-length cross-linking agents eliminate the potential for this type of cross-reactivity by mediating a direct linkage between two substances.

Homobifunctional reagents, which were the first cross-linking reagents used for modification and conjugation of macromolecules, consisted of bireactive compounds containing the same functional group at both ends (Hartman and Wold, 1965). These reagents could tie one protein to another by covalently reacting with the same common groups on both molecules. Thus, the lysine  $\epsilon$ -amines or N-terminal amines of one protein could be cross-linked to the same functional groups on a second protein simply by mixing the two together in the presence of the homobifunctional reagent.

Heterobifunctional conjugation reagents contain two different reactive groups that can couple to two different functional targets on proteins and other macromolecules. For example, one part of a cross-linker may contain an amine-reactive group, while another portion may consist of a sulphydryl-reactive group. The result is the ability to

5 direct the cross-linking reaction to selected parts of target molecules, thus garnering better control over the conjugation process.

Heterobifunctional reagents are used to cross-link proteins and other molecules in a two- or three-step process that limits the degree of polymerization often obtained

10 using homobifunctional cross-linkers.

Many methods are currently available for coupling of peptide immunogens to protein/polypeptide carriers using zero-length, homobifunctional or heterobifunctional crosslinkers. Most methods create amine, amide, urethane, isothiourea, or disulfide

15 bonds, or in some cases thioethers. The more general method of coupling proteins or peptides to peptides utilizes bifunctional crosslinking reagents. These are small spacer molecules having active groups at each end. The spacer molecules can have identical or different active groups at each end. The most common active functionalities, coupling groups, and bonds formed are:

20 1. Aldehyde - amino → secondary amine  
2. Maleimido - sulphydryl → thioether  
3. Succinimido - amino → amide  
4. Imidate esters - amino → amide  
5. Phenyl azides - amino → phenyl amine  
25 6. Acyl halide - sulphydryl → thioether  
7. Pyridyldisulfides - sulphydryl → disulfide  
8. Isothiocyanate - amino → isothiourea.

The reactivity of a given carrier protein, in terms of its ability to be modified by a cross-linking agent such that it can be conjugated to an peptide immunogen, is

30 determined by its amino acid composition and the sequence location of the individual amino acids in the three dimensional structure of the molecule, as well as by the amino acid composition of the peptide immunogen.

In the case of linkers ("L") between protein/peptide carriers and other peptides (e.g., a protein/peptide carriers and an peptide immunogen), the spacers are typically selected from Ala, Gly, or other neutral spacers of nonpolar amino acids or neutral polar amino acids. In certain embodiments the neutral spacer is Ala. It will be

5 understood that the optionally present spacer need not be comprised of the same residues and thus may be a hetero- or homo-oligomer. Exemplary spacers include homo-oligomers of Ala. When present, the spacer will usually be at least one or two residues, more usually three to six residues. In other embodiments the protein/polypeptide carrier is conjugated to an peptide immunogen, preferably with

10 the protein/peptide carrier positioned at the amino terminus. The peptide may be joined by a neutral linker, such as Ala-Ala-Ala or the like, and preferably further contain a lipid residue such palmitic acid or the like which is attached to alpha and epsilon amino groups of a Lys residue ((PAM)<sub>2</sub>Lys), which is attached to the amino terminus of the peptide conjugate, typically via Ser-Ser linkage or the like.

15 In some aspects of the invention, the peptide immunogen is an A $\beta$  fragment selected from the group consisting of A $\beta$ 1-5-L, A $\beta$ 1-7-L, A $\beta$ 1-9-L, and A $\beta$ 1-12-L. In some aspects of the invention the linker is GACA (SEQ ID NO:10).

To facilitate the conjugation of a peptide immunogen with a carrier, additional amino acids can be added to the termini of the antigenic determinants. The additional residues can also be used for modifying the physical or chemical properties of the peptide immunogen. Amino acids such as tyrosine, cysteine, lysine, glutamic or aspartic acid, or the like, can be introduced at the C- or N- terminus of the peptide immunogen. Additionally, peptide linkers containing amino acids such as glycine and alanine can also be introduced. In addition, the antigenic determinants can differ from the natural sequence by being modified by terminal NH<sub>2</sub>-group acylation, e.g., by alkanoyl (C1-C20) or thioglycolyl acetylation, terminal-carboxy amidation, e.g., ammonia, methylamine, etc. In some instances these modifications may provide sites for linking to a support or other molecule.

20 25 30 In some aspects of the invention, the peptide immunogen is an A $\beta$  fragment selected from the group consisting of A $\beta$ 1-5-C, A $\beta$ 1-7-C, A $\beta$ 1-9-C, and A $\beta$ 1-12-C, where C is a cysteine amino acid residue. In some aspects of the invention, the peptide

immunogen is an A $\beta$  fragment selected from the group consisting of A $\beta$ 1-5-L-C, A $\beta$ 1-7-L-C, A $\beta$ 1-9-L-C, and A $\beta$ 1-12-L-C.

The peptide immunogen is linked to the protein/peptide carrier either directly or via a linker either at the amino or carboxy terminus of the peptide immunogen.

5 The amino terminus of either the peptide immunogen or the protein/peptide carrier may be acylated. In addition, the peptide immunogen -protein/peptide carrier conjugate may be linked to certain alkanyl (C<sub>1</sub>-C<sub>20</sub>) lipids via one or more linking residues such as Gly, Gly-Gly, Ser, Ser-Ser as described below. Other useful lipid moieties include cholesterol, fatty acids, and the like.

10

Peptide immunogens can be linked to a carrier by chemical crosslinking. Techniques for linking an immunogen to a carrier include the formation of disulfide linkages using N-succinimidyl-3-(2-pyridyl-thio) propionate (SPDP) (Carlsson, J *et al.* (1978) *Biochem J*, 173: 723,) and succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-

15 carboxylate (SMCC) (if the peptide lacks a sulphydryl group, this can be provided by addition of a cysteine residue to the hapten). These reagents create a disulfide linkage between themselves and peptide cysteine resides on one protein and an amide linkage through the  $\epsilon$ -amino on a lysine, or other free amino group in other amino acids. A variety of such disulfide/amide-forming agents are described in  
20 *Immune. Rev.* 62: 85 (1982). Other bifunctional coupling agents form a thioether rather than a disulfide linkage. The thioether forming agents include reactive ester of 6-maleimidocaproic acid, 2-bromoacetic acid, and 2-iodoacetic acid, 4-(N-maleimido-methyl) cyclohexane-1-carboxylic acid. The carboxyl groups can be activated by combining them with succinimide or 1-hydroxyl-2-nitro-4-sulfonic acid,  
25 sodium salt.

Most frequently, lysine residues are the most abundant amino acid residues found on carrier proteins, and these residues are modified using cross-linking reagents to generate nucleophilic sites that are then coupled to a hapten. This coupling is

30 achieved via any of the hydrophilic side chains on the hapten molecules that are chemically active. These include the guanidyl group of arginine, the  $\gamma$ -carboxyl groups of glutamate and aspartic acid, the sulphydryl group of cysteine, and the  $\epsilon$ -amino group of lysine, to name a few. Modification of proteins such that they can

now be coupled to other moieties is achieved using crosslinking reagents, which react with any of the side chains on the protein carrier or hapten molecules.

In one aspect of the present invention, the carrier protein with or without a linker molecule is functionalized (derivatized) with a reagent that introduces reactive sites into the carrier protein molecule that are amenable to further modification to introduce nucleophilic groups. In one embodiment, the carrier is reacted with a haloacetyating reagent, which preferentially reacts with a number of functional groups on amino acid residues of proteins such as the sulphydryl group of cysteine, the primary  $\epsilon$ -amine group of lysine residue, the  $\alpha$  terminal of  $\alpha$ -amines, the thioether of methionine and both imidazoyl side chain nitrogens of histidine (Gurd, 1967). In a preferred embodiment, the primary  $\epsilon$ -amine groups on lysine residues of the carrier protein are derivatized with  $\beta$ -N-hydroxysuccinimidyl bromoacetate to generate a bromoacetylated carrier. Conjugation of peptide immunogen and the activated protein carrier was carried out by slowly adding the activated carrier to the solution containing the peptide immunogen.

By using the process of this invention, the peptide immunogens discussed in section B, above, may be conjugated to any of the carriers discussed in section A, above. The conjugates resulting from the process of this invention are used as immunogens for the generation of antibodies against A $\beta$  for use in passive/active immunotherapy. Furthermore, A $\beta$  or an A $\beta$  fragment linked to a carrier can be administered to a laboratory animal in the production of monoclonal antibodies to A $\beta$ .

In one aspect of the invention, the conjugate is a conjugate selected from the group consisting of A $\beta$ 1-7-CRM<sub>197</sub>, (A $\beta$ 1-7 x 3)-CRM<sub>197</sub>, and (A $\beta$ 1-7 x 5)-CRM<sub>197</sub>. In one aspect of the invention, the conjugate is a conjugate selected from the group consisting of CRM<sub>197</sub>-A $\beta$ 1-5, CRM<sub>197</sub>-A $\beta$ 1-7, CRM<sub>197</sub>-A $\beta$ 1-9, and CRM<sub>197</sub>-A $\beta$ 1-12. In another aspect of the invention, the conjugate is a conjugate selected from the group consisting of A $\beta$ 1-5-C-CRM<sub>197</sub>, A $\beta$ 1-7-C-CRM<sub>197</sub>, A $\beta$ 1-9-C-CRM<sub>197</sub>, and A $\beta$ 1-12-C-CRM<sub>197</sub>. In yet another aspect of the invention, the conjugate is a conjugate

selected from the group consisting of selected from the group consisting of A $\beta$ 1-5-L-C-CRM<sub>197</sub>, A $\beta$ 1-7-L-C-CRM<sub>197</sub>, A $\beta$ 1-9-L-C-CRM<sub>197</sub>, and A $\beta$ 1-12-L-C-CRM<sub>197</sub>.

#### D. Capping

A disadvantage to the use of coupling reagents, which introduce reactive sites into the side chains of reactive amino acid molecules on carrier and /or hapten molecules, is that the reactive sites if not neutralized are free to react with any unwanted molecule either *in vitro* or *in vivo*. In the process of the present invention, capping of the unreacted functional groups is accomplished by reaction of the conjugates with pendant reactive groups with reagents which inactivate/cap the reactive groups.

Exemplary inactivating/capping reagents for use with the conjugation process of the present invention include cysteamine, *N*-acetylcysteamine, and ethanolamine. Alternatively, capping is accomplished by reaction with ammonia or ammonium bicarbonate, either of which converts the haloacetyl groups to aminoacetyl groups. Capping is also accomplished at alkaline pH (9.0–9.8) using sodium hydroxide or sodium carbonate, which converts the haloacetyl groups to hydroxyacetyl groups. One potential advantage of converting the haloacetyl groups to aminoacetyl or hydroxyacetyl groups, as opposed to the reaction with cysteamine derivatives, ethanolamine etc., is the introduction of relatively smaller size chemical functionalities, by reaction with ammonia or hydroxide/carbonate. The resulting capped functional groups, e.g. aminoacetyl or hydroxyacetyl, provide relatively less perturbation in the carrier protein portion of the conjugate. The capped peptide immunogen-carrier protein is purified as necessary using known methods, such as chromatography (gel filtration, ion exchange, hydrophobic interaction or affinity), dialysis, ultrafiltration-diafiltration, selective precipitation using ammonium sulfate or alcohol, and the like.

#### E. Immunogenic Conjugates and Compositions

The capped peptide immunogen-carrier protein conjugates are administered in an immunogenic composition to mammals, particularly humans, for prophylactic and/or therapeutic purposes. The conjugates of the present invention are used to elicit and/or enhance immune responses against immunogens. For instance, CTL-carrier conjugates are used to treat and/or prevent viral infection, amyloidogenic diseases, cancer etc. Alternatively, polypeptide immunogen-carrier conjugates, which induce

antibody responses, are also used. Examples of diseases, which can be treated using the conjugates of the present invention, include various bacterial infections, viral infections, fungal infections, parasitic infections and cancer.

In therapeutic applications, a conjugate of the present invention is administered to an individual already suffering from an amyloidogenic disease such as Alzheimer's disease, cancer, or infected with a pathogenic microorganism. Those in the incubation phase or the acute phase of the disease may be treated with the conjugate of the present invention separately or in conjunction with other treatments, as appropriate.

5        In therapeutic applications, an immunogenic composition of the present invention is administered to a patient in an amount sufficient to elicit an effective CTL response or humoral response to the microorganism, amyloid plaque or to a tumor antigen expressed on a cancer cell, and to cure, or at least partially arrest disease progression, symptoms and/or complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend in part on the peptide composition, the manner of administration, the stage and severity of the disease being treated, the weight and general state of health of the patient, and the judgment of the prescribing physician.

10      Therapeutically effective amounts of the immunogenic compositions of the present invention generally range for the initial immunization for therapeutic or prophylactic administration, from about 0.1  $\mu$ g to about 10,000  $\mu$ g of peptide for a 70 kg patient, usually from about 0.1 to about 8000  $\mu$ g, preferably between about 0.1 to about 5000  $\mu$ g, and most preferably between 0.1 to about 1,000  $\mu$ g. These doses are followed by boosting dosages of from about 0.1  $\mu$ g to about 1000  $\mu$ g of peptide pursuant to a boosting regimen over weeks to months depending upon the patient's response and condition by measuring specific immune responses.

15      Further, the present invention is used prophylactically to prevent and/or ameliorate bacterial infections, viral infections, fungal infections, parasitic infections, amyloidogenic disease, or cancer. Effective amounts are as described above.

20      Additionally, one of ordinary skill in the art would also know how to adjust or modify

prophylactic treatments, as appropriate, for example by boosting and adjusting dosages and dosing regimes.

Therapeutic administration may begin at the first sign of the disease or the detection or surgical removal of tumors or shortly after diagnosis in the case of acute infection.

5     This is followed by boosting doses until the disease progression is halted or reversed or the symptoms are substantially abated and for a period thereafter. In chronic infection, initial high doses followed by boosting doses may be required.

Treatment of an infected individual with the compositions of the invention may hasten resolution of the infection in acutely infected individuals. For those individuals

10    susceptible (or predisposed) to developing chronic infection the compositions are particularly useful in methods for preventing the evolution from acute to chronic infection. Where the susceptible individuals are identified prior to or during infection, for instance, as described herein, the composition can be targeted to them, minimizing need for administration to a larger population.

15    The conjugates of the present invention are also used for the treatment of chronic infection and to stimulate the immune system to eliminate virus-infected cells in individuals with latent infections. It is important to provide an amount of the immunogenic composition of the present invention in a formulation and mode of administration sufficient to effectively elicit and/or enhance an immune response.

20    Thus, for treatment of chronic infection, a representative dose is in the range from about 0.1  $\mu$ g to about 10,000  $\mu$ g of peptide, usually from about 0.1 to about 8000  $\mu$ g, preferably between about 0.1 to about 5000  $\mu$ g, and most preferably between 0.1 to about 1,000  $\mu$ g for a 70kg patient per dose. Immunizing doses followed by boosting doses at established intervals, e.g., from one to four weeks, may be required, 25    possibly for a prolonged period of time to effectively immunize an individual. In the case of chronic infection, administration should continue until at least clinical symptoms or laboratory tests indicate that the viral infection has been eliminated or substantially abated and for a period thereafter.

30    Immunogenic compositions of the present invention for therapeutic or prophylactic treatment can be administered by parenteral, topical, intravenous, oral, subcutaneous, intra-arterial, intra-cranial, intra-peritoneal, intra-nasal or intra-

muscular means for prophylactic and/or therapeutic treatment. One typical route of administration of an immunogenic agent is subcutaneous, although other routes can be equally effective. Another common route is intra-muscular injection. This type of injection is most typically performed in the arm or leg muscles. In some methods, 5 agents are injected directly into a particular tissue where deposits have accumulated, for example intra-cranial injection. Intra-muscular injection or intravenous infusion is preferred for administration of antibody. In some methods, particular therapeutic antibodies are injected directly into the cranium. Because of the ease of administration, the immunogenic compositions of the invention are particularly 10 suitable for oral administration. The invention further provides immunogenic compositions for parenteral administration, which comprise a solution of the peptides or conjugates, dissolved or suspended in an acceptable carrier, preferably an aqueous carrier.

A variety of diluents, excipients and buffers may be used, e.g., water, buffered water, 15 phosphate buffered saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional, well-known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable 20 auxiliary substances as required to approximate physiological conditions, such as buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

For solid compositions, conventional nontoxic solid carriers may be used. These may 25 include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active 30 ingredient, that is, one or more conjugates of the invention, and more preferably at a concentration of 25-75%.

The concentration of immunogenic compositions of the present invention in the pharmaceutical formulations can vary widely, i.e., from less than about 0. 1%, usually at or at least about 2% to as much as 20% to 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, etc. , in accordance with the particular 5 mode of administration selected.

The conjugates of the present invention may also be administered via liposomes, which serve to target the conjugates to a particular tissue, such as lymphoid tissue, or targeted selectively to infected cells, as well as increase the half-life of the peptide 10 composition. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In these preparations the composition to be delivered is incorporated as part of a liposome, alone or in conjunction with a molecule, which binds to, for example, a receptor prevalent among lymphoid cells. These molecules would include monoclonal antibodies, which bind to the CD45 antigen, or with other therapeutic or immunogenic 15 compositions. Thus, liposomes filled with a desired composition of the present invention can be directed to the site of lymphoid cells, where the liposomes then deliver the selected therapeutic/immunogenic peptide compositions. Liposomes for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as 20 cholesterol. The selection of lipids is generally guided by consideration of liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, e.g., Szoka, et al., *Ann. Rev. Biophys. Bioeng.* 9:467 (1980), U.S. Pat. Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369, incorporated herein by reference.

25 For targeting to the immune cells, a ligand to be incorporated into the liposome can include antibodies or fragments thereof specific for cell surface determinants of the desired immune system cells. A liposome suspension containing a composition of the present invention may be administered intravenously, locally, topically, etc. in a dose which varies according to, *inter alia*, the manner of administration, the composition 30 being delivered, and the stage of the disease being treated.

For aerosol administration, the compositions of the present invention are preferably supplied in finely divided form along with a surfactant and propellant. Typical

percentages of the composition are 0.01-20% by weight, preferably 1-10%. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, 5 stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute 0.1- 20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. A carrier can also be included, if desired, as with lecithin for intranasal delivery.

10 The compositions of this invention may also be used to make monoclonal antibodies. Such antibodies may be useful as potential diagnostic or therapeutic agents.

The compositions of the present invention may also find use as diagnostic reagents. For example, a composition of the invention may be used to determine the susceptibility of a particular individual to a treatment regimen, which employs the 15 polypeptide immunogens, and thus may be helpful in modifying an existing treatment protocol or in determining a prognosis for an affected individual. In addition, the compositions of the present invention may also be used to predict which individuals will be at substantial risk for developing chronic infection.

Conjugates of the present invention can optionally be administered in combination 20 with other agents that are at least partly effective in treatment and/or amelioration of a disease and/or its symptoms. In the case of Alzheimer's and Down's syndrome, in which amyloid deposits occur in the brain, the conjugates of the invention can be administered in conjunction with other agents that increase passage of the agents of the invention across the blood-brain barrier.

25 The immunogenic composition typically contains an adjuvant. An adjuvant is a substance that enhances the immune response when administered together with an immunogen or antigen. A number of cytokines or lymphokines have been shown to have immune modulating activity, and thus may be used as adjuvants, including, but 30 not limited to, the interleukins 1- $\alpha$ , 1- $\beta$ , 2, 4, 5, 6, 7, 8, 10, 12 (see, e.g., U.S. Patent No. 5,723,127), 13, 14, 15, 16, 17 and 18 (and its mutant forms), the interferons- $\alpha$ ,  $\beta$

and  $\gamma$ , granulocyte-macrophage colony stimulating factor (see, e.g., U.S. Patent No. 5,078,996) macrophage colony stimulating factor, granulocyte colony stimulating factor, GSF, and the tumor necrosis factor  $\alpha$  and  $\beta$ . Still other adjuvants useful in this invention include a chemokine, including without limitation, MCP-1, MIP-1 $\alpha$ , MIP-1 $\beta$ , 5 and RANTES. Adhesion molecules, such as a selectin, e.g., L-selectin, P-selectin and E-selectin may also be useful as adjuvants. Still other useful adjuvants include, without limitation, a mucin-like molecule, e.g., CD34, GlyCAM-1 and MadCAM-1, a member of the integrin family such as LFA-1, VLA-1, Mac-1 and p150.95, a member of the immunoglobulin super family such as PECAM, ICAMs, e.g., ICAM-1, ICAM-2 10 and ICAM-3, CD2 and LFA-3, co-stimulatory molecules such as CD40 and CD40L, growth factors including vascular growth factor, nerve growth factor, fibroblast growth factor, epidermal growth factor, B7.2, PDGF, BL-1, and vascular endothelial growth factor, receptor molecules including Fas, TNF receptor, Flt, Apo-1, p55, WSL-1, DR3, TRAMP, Apo-3, AIR, LARD, NGRF, DR4, DR5, KILLER, TRAIL-R2, TRICK2, and 15 DR6. Still another adjuvant molecule includes Caspase (ICE). See, also International Patent Publication Nos. WO98/17799 and WO99/43839, incorporated herein by reference.

Suitable adjuvants used to enhance an immune response include, without limitation, 20 MPL<sup>TM</sup> (3-O-deacylated monophosphoryl lipid A; Corixa, Hamilton, MT), which is described in U.S. Patent No. 4,912,094, which is hereby incorporated by reference. Also suitable for use as adjuvants are synthetic lipid A analogs or aminoalkyl glucosamine phosphate compounds (AGP), or derivatives or analogs thereof, which are available from Corixa (Hamilton, MT), and which are described in U.S. Patent No. 25 6,113,918, which is hereby incorporated by reference. One such AGP is 2-[(R)-3-Tetradecanoyloxytetradecanylamino] ethyl 2-Deoxy-4-O-phosphono-3-O-[(S)-3-tetradecanoyloxytetradecanoyl]-2-[(R)-3-tetradecanoyloxy-tetradecanoyl-amino]-b-D-glycopyranoside, which is also known as 529 (formerly known as RC529). This 529 adjuvant is formulated as an aqueous form or as a 30 stable emulsion.

Still other adjuvants include mineral oil and water emulsions, calcium salts such as calcium phosphate, aluminum salts (alum), such as aluminum hydroxide, aluminum

phosphate, etc., Amphigen, Avridine, L121/squalene, D-lactide-polylactide/glycoside, pluronic acids, polyols, muramyl dipeptide, killed *Bordetella*, saponins, such as Stimulon™ QS-21 (Antigenics, Framingham, MA), described in U.S. Patent No. 5,057,540, which is hereby incorporated by reference, and particles generated 5 therefrom such as ISCOMS (immunostimulating complexes), *Mycobacterium tuberculosis*, bacterial lipopolysaccharides, synthetic polynucleotides such as oligonucleotides containing a CpG motif (U.S. Patent No. 6,207,646, which is hereby incorporated by reference), a pertussis toxin (PT), or an *E. coli* heat-labile toxin (LT), particularly LT-K63, LT-R72, PT-K9/G129; see, e.g., International Patent Publication 10 Nos. WO 93/13302 and WO 92/19265, incorporated herein by reference.

Also useful as adjuvants are cholera toxins and mutants thereof, including those described in published International Patent Application number WO 00/18434 (wherein the glutamic acid at amino acid position 29 is replaced by another amino 15 acid (other than aspartic acid, preferably a histidine). Similar CT toxins or mutants are described in published International Patent Application number WO 02/098368 (wherein the isoleucine at amino acid position 16 is replaced by another amino acid, either alone or in combination with the replacement of the serine at amino acid position 68 by another amino acid; and/or wherein the valine at amino acid position 20 72 is replaced by another amino acid). Other CT toxins are described in published International Patent Application number WO 02/098369 (wherein the arginine at amino acid position 25 is replaced by another amino acid; and/or an amino acid is inserted at amino acid position 49; and/or two amino acids are inserted at amino acid position 35 and 36).

25 It is to be understood that reference throughout this specification to any theory to explain the results described is not to limit the scope of the invention. Independent of the method by which the invention functions, the results and advantages described herein may be achieved by reference to the following examples of the invention.

30

**EXAMPLE 1****CONJUGATION OF CRM<sub>197</sub> TO A $\beta$  PEPTIDE**

Conjugation of haptens/antigenic peptides was carried out by reacting activated 5 carrier CRM<sub>197</sub>, which has thirty-nine lysine residues, to a hapten/antigenic peptide having a pendant thiol-group using the method described below (Figure 1). All the A $\beta$  peptides contained a cysteine residue at the carboxy terminus to facilitate the conjugation of these peptides through the cysteinyl sulfhydryl group to the carrier protein. These peptides were produced by solid phase synthesis.

**10 I. Activation**

Free amino groups of CRM<sub>197</sub> were bromoacetylated by reaction with an excess of bromoacetic acid *N*-hydroxysuccinimide ester (Sigma Chemical Co., St. Louis, MO) (Bernatowicz and Matsueda, 1986). To an ice-cold solution of CRM<sub>197</sub> (~15 mg), 10% (v/v) 1.0 M NaHCO<sub>3</sub> (pH 8.4) was added. Bromoacetic acid *N*-hydroxysuccinimide ester, equal in weight to that of CRM<sub>197</sub> used, was dissolved in 200  $\mu$ L dimethylformamide (DMF), added slowly to the CRM<sub>197</sub>, and gently mixed at room temperature in the dark for 1 hour. The resulting bromoacetylated (activated) 15 protein was purified by passage through a desalting (P6-DG) column using PBS / 1 mM EDTA (pH 7.0) as the eluent. Following purification, the fractions corresponding to activated CRM<sub>197</sub> were pooled and the protein concentration was estimated by 20 BCA protein assay. The protein amino groups, both before and after treatment with bromoacetic acid *N*-hydroxysuccinimide ester, were reacted with 2,4,6-trinitrobenzenesulfonic acid (TNBSA), which served as an indicator of bromoacetylation (Means *et al.*, 1972).

**25 II. Conjugation**

Prior to conjugation, the peptides were reacted with 5,5'-dithio-bis(2-nitrobenzoic acid) [Ellman's reagent] to verify the content of free -SH groups (between 62-88% reduced). For the first four A $\beta$  peptides (amino acids 1-7 without linker, amino acids 1-12 with GAGAC linker, amino acids 1-9 with GAGAC linker, and amino acids 1-7 30 with GAGAC linker), approximately 8.0 – 10.0 mg of peptide was dissolved in sterile distilled water to an approximate concentration of 20 mg/ml. The peptide was slowly added to cold activated CRM<sub>197</sub> in a 1:1 ratio (w/w) and the pH was adjusted to

approximately 7.0-7.2 with the addition of 20-36  $\mu$ l of 1 N NaOH. The resulting material was gently mixed overnight at 4°C in the dark followed by dialysis in the dark against two 1L changes of PBS, pH 7.2. For the next four A $\beta$  peptides (amino acids 1-5 without linker, amino acids 1-9 without linker, amino acids 1-12 without linker, and amino acids 1-5 with linker), reaction with Ellman's reagent was used to verify the free -SH groups. CRM<sub>197</sub> was bromoacetylated, purified, and reacted with TNBSA as previously described. The pH of each peptide was adjusted to 7.0 with the addition of 0.1 M NaPO<sub>4</sub> (pH 8.5) at 2.2x the volume of the dissolved peptide. The peptide was slowly added to cold activated CRM<sub>197</sub> in a 1:1 ratio and allowed to react overnight at 4°C in the dark. The resulting material was dialyzed. A final control peptide (1-12mer in reverse orientation) was conjugated to CRM<sub>197</sub> as described above with the following modification. Rather than adjusting the pH of the peptide to 7.0, the pH of the activated CRM<sub>197</sub> was adjusted to approximately 7.5 with the addition of 20% (v/v) 0.5 M NaPO<sub>4</sub> (pH 8.0). Each conjugate, after dialysis, was transferred into a sterile 15mL polypropylene tube, wrapped in aluminum foil, and stored at 4°C. Activation of the reactive amino residues on the carrier was then subsequently verified using mass spectrometry.

Conjugate	Immunogenic Peptide
A $\beta$ 1-5-C-CRM <sub>197</sub>	DAEFR-C (SEQ. ID. NO.:1)
A $\beta$ 1-7-C-CRM <sub>197</sub>	DAEFRHD-C (SEQ. ID NO.:2)
A $\beta$ 1-9-C-CRM <sub>197</sub>	DAEFRHDSG-C (SEQ ID NO:3)
A $\beta$ 1-12-C-CRM <sub>197</sub>	DAEFRHDSGYEV-C (SEQ ID NO:4)
A $\beta$ 1-5-L-C-CRM <sub>197</sub>	DAEFR-GAGA-C (SEQ ID NO.:5)
A $\beta$ 1-7-L-C-CRM <sub>197</sub>	DAEFRHD-GAGA-C (SEQ ID NO.:6)
A $\beta$ 1-9-L-C-CRM <sub>197</sub>	DAEFRHDSG-GAGA-C (SEQ ID NO.:7)
A $\beta$ 1-12-L-C-CRM <sub>197</sub>	DAEFRHDSGYEV-GAGA-C (SEQ ID NO.:8)
A $\beta$ 12-1-C-CRM <sub>197</sub> (-VE CONTROL)	VEYGSDHRFEAD-C (SEQ ID NO.: 9)

L= linker (GAGA) (SEQ ID NO.:10)

## EXAMPLE 2

### 5 PREPARATION OF A $\beta$ PEPTIDE-CRM<sub>197</sub> CONJUGATE AND PURIFICATION BY ULTRAFILTRATION

#### I. Bromoacetylation of CRM<sub>197</sub>

CRM<sub>197</sub> (100 mg) in 0.01 M sodium phosphate buffer, 0.9% NaCl, pH 7.0, was  
10 reacted with bromoacetic acid *N*-hydroxysuccinimide ester (dissolved to 20 mg/mL in  
DMSO) at a 1:1 weight ratio under an argon atmosphere. The reaction was titrated  
as needed to maintain the pH at 7.0. The mixture was stirred in dark for 1.5 hours at  
room temperature. The reaction mixture was 1.2 $\mu$ m filtered into the retentate  
reservoir of a UF/DF system (Millipore Labscale TFF, Billerica, MA). Purification was  
15 done using a 10K or 30K UF membrane by diafiltration (30-fold) against 0.01 M  
sodium phosphate buffer / 0.9% NaCl, pH 7.0. The bromoacetylated CRM<sub>197</sub> was  
filtered by passing through a 0.2 $\mu$ m filter. The degree of bromoacetylation was  
determined by reacting the activated CRM<sub>197</sub> with cysteine, followed by amino acid  
analysis and quantitation of the resulting carboxymethylcysteine (CMC).

#### 20 II. Conjugation of A $\beta$ Peptide and Bromoacetylated CRM<sub>197</sub> and Capping with N- acetylcysteamine

Bromoacetylated CRM<sub>197</sub> (50 mg) was transferred to a reaction vessel. To the stirred  
solution, maintained at 2-8°C, was added 1 M sodium carbonate/bicarbonate.  
Titration was performed to achieve a target pH of 9.0, under argon atmosphere.  
25 Separately, 50 mg of A $\beta$  peptide was weighed out and dissolved in water for injection  
(WFI) to 20 mg/mL. To this solution was added 1 M sodium carbonate/bicarbonate  
until pH 9.0 was attained. The peptide solution was added to the bromoacetylated  
CRM<sub>197</sub> solution, and the mixture was stirred at 2-8°C for 14-18 hours. The  
remaining bromoacetyl groups were capped with a 20-fold molar excess of *N*-  
30 acetylcysteamine for 3-6 hours at 2-8°C.

The reaction mixture was filtered through 1.2 $\mu$ m filter into the retentate reservoir of a UF/DF system (Millipore XL), and the conjugate was purified at room temperature by 30-fold diafiltration on a 10K or 30K MWCO membrane (Millipore) by diafiltering against 0.01 M sodium phosphate buffer / 0.9% NaCl, pH 7.0. The retentate was 5 collected and 0.2 $\mu$ m filtered and analyzed for protein content (Lowry or Micro-BCA colorimetric assay), by SDS-PAGE, by amino acid analysis, and for immunogenicity in mice.

#### 10 EXAMPLE 3

##### **CONVERSION BY CAPPING OF THE UNREACTED BROMOACETYL GROUPS TO AMINOACETYL GROUPS**

Bromoacetylated CRM<sub>197</sub> (50 mg), prepared as described above in example 2, was 15 transferred to a reaction vessel. To the stirred solution, maintained at 2-8°C, was added 1M sodium carbonate/bicarbonate. Titration was performed to achieve a target pH of 9.0, under argon atmosphere. Separately, 50 mg of A $\beta$  peptide was weighed out and dissolved in WFI to 20 mg/mL. To this solution was added 1 M sodium carbonate/bicarbonate until pH 9.0 was attained. The peptide solution was 20 added to the bromoacetylated CRM<sub>197</sub> solution, and the mixture was stirred at 2-8°C for 14-18 hours. The remaining bromoacetyl groups were capped using 8% ammonium bicarbonate solution for 4 hours at 2-8°C.

The reaction mixture was 1.2 $\mu$ m filtered into the retentate reservoir of a UF/DF 25 system (Millipore XL), and the conjugate was purified at room temperature by 30-fold diafiltration on a 10K or 30K MWCO membrane by diafiltering vs 0.01 M sodium phosphate buffer / 0.9% NaCl, pH 7.0. The retentate was collected and 0.2 $\mu$ m filtered and analyzed for protein content (Lowry or Micro-BCA colorimetric assay), by SDS-PAGE, by amino acid analysis, and for immunogenicity in mice.

30

## EXAMPLE 4

QUANTITATIVE DETERMINATION OF S-CARBOXYMETHYLCYSTEINE AND S-CARBOXYMETHYLCYSTEAMINE AS EVALUATION OF DEGREE OF CONJUGATION AND CAPPING OF PEPTIDE IMMUNOGENS-  
5 PROTEIN/POLYPEPTIDE CONJUGATES

Acid hydrolysis of protein-peptide conjugates generated using bromoacetyl activation chemistry resulted in the formation of acid stable S-carboxymethylcysteine (CMC) from the cysteines at the conjugated sites and the formation of acid stable S-carboxymethylcysteamine (CMCA) from the cysteamine at the capped sites (Figure 10 2). All of the conjugated and capped Lysines were converted back to Lysine and detected as such. All other amino acids were hydrolyzed back to free amino acids except for Tryptophan and Cysteine, which were destroyed by the hydrolysis conditions. Asparagine and Glutamine were converted to Aspartic Acid and Glutamic Acid respectively.

15 Conjugate samples were diluted with deionized water to a total protein concentration of less than 1 mg/mL. Two 10 microgram aliquots of each conjugate were dried and resuspended in 100  $\mu$ L of 6N HCl [Pierce], 5  $\mu$ L of melted phenol [Sigma-Aldrich], and 1  $\mu$ L of 2-mercaptoethanol [Sigma-Aldrich]. The samples were then incubated 20 under vacuum (100 mT) at 110 °C for 22 hours. The resulting hydrolysates were dried, resuspended in 250  $\mu$ L of Beckman Na-S sodium citrate sample dilution buffer (pH 2.2) [Beckman Instruments, Inc., Fullerton, CA], and filtered using Whatman 0.2  $\mu$ m nylon syringe tip filters and 1mL syringes.

25 Each sample was then loaded into a Beckman 6300 amino acid analyzer sample loop and placed in the analyzer. The amino acids of each hydrolyzed sample and control were separated using ion exchange chromatography followed by reaction with Beckman Ninhydrin NinRX solution at 135 °C. The derivatized amino acids were then detected in the visible range at 570 nm and 440 nm (see Table 1). A standard 30 set of amino acids [Pierce Amino Acid Standard H] containing 500 picomoles of each amino acid was run along with the samples and controls for each set of analysis. S-carboxymethylcysteine [Sigma-Aldrich] was added to the standard.

Table 1 – Retention Times for Amino Acids using Gradient Program 1 on the Beckman 6300 Amino Acid Analyzer

Retention Time (min.)	Amino Acid	Wavelength used for Detection
8.3	Carboxymethylcysteine	CMC
9.6	Aspartic Acid & Asparagine	Asx
11.3	Threonine	Thr
12.2	Serine	Ser
15.8	Glutamic Acid & Glutamine	Glx
18.5	Proline	Pro
21.8	Glycine	Gly
23.3	Alanine	Ala
29.0	Valine	Val
32.8	Methionine	Met
35.5	Isoleucine	Ile
36.8	Leucine	Leu
40.5	Tyrosine	Tyr
42.3	Phenylalanine	Phe
45.4	Carboxymethylcysteamine	CMCA
48.8	Histidine	His
53.6	Lysine	Lys
70.8	Arginine	Arg

5 The areas of each standard peak were used as a quantitative equivalence for proportional evaluation of each sample. Proline was determined from 440 nm and was converted to an equivalence in 570 nm using Glutamic acid, the closest amino acid.

10 Each of these picomole values was converted to a molar ratio of amino acid residues using a comparison of picomoles of lysine to the theoretical lysine value present in the protein. Lysine was chosen for this evaluation based on its covalent attachment

to Cysteine and Cysteamine and the expected similar hydrolysis. The resulting numbers of moles of amino acids were then compared to the amino acid composition of the protein and reported along with the values for CMC and CMCA. The CMC value was used directly for evaluation of the degree of conjugation and the CMCA value was used directly for evaluation of the degree of capping.

5 **EXAMPLE 5**

**CHARACTERIZATION AND OPTIMIZATION OF A<sub>β</sub>-CRM<sub>197</sub> PEPTIDE CONJUGATES**

To verify conjugation, all peptide-CRM<sub>197</sub> conjugates were analyzed by amino acid analysis and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF)mass spectrometry. For each conjugate, the moles of peptide conjugated to each mole CRM<sub>197</sub> was determined by amino acid analysis (number of S-carboxymethylcysteine residues) and MALDI-TOF mass spectrometry. The values determined by each method were generally in agreement.

15

**I. Size exclusion chromatography:**

Batch concentrate samples were removed from storage and allowed to warm to room temperature. The A<sub>β</sub> peptide conjugate sample was gently mixed to insure a homogeneous preparation. The A<sub>β</sub> peptide conjugate sample was spun in an 20 Eppendorf micro-centrifuge to remove any particulates. The supernatant was withdrawn for TosoHaas TSK-Gel G3000SW chromatography (TosoHaas, Stuttgart, Germany). A TosoHaas TSK-Gel G3000SW column was connected to a HPLC system and the pressure limit was set to 1.4 MPa. The column was equilibrated with at least 30 mL of PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.2 ± 0.1) at a 25 flow rate of 0.75 mL/min. The A<sub>β</sub> peptide conjugate sample was loaded onto the TosoHaas TSK-Gel G3000SW column using the following parameters:

- Concentration of A<sub>β</sub> peptide conjugate sample: 1.5 ± 1.0 mg/mL
- Flow rate: 0.75 mL/min
- Sample Volume: 0.1 mL
- 30 Run Time: 30 minutes

The absorbance was monitored at both 280nm and 210nm. For long term storage, the TosoHaas TSK-Gel G3000SW column was equilibrated with at least 50 mL of 20% ethanol at a flow rate of 0.5 – 1.0 mL/min.

5     **II. PAGE (Polyacrylamide Gel Electrophoresis):**

The activated (bromoacetylated) CRM<sub>197</sub> and the A $\beta$  peptide-CRM<sub>197</sub> conjugates were examined by SDS-Gels using a NuPAGE Bis-Tris Electrophoresis (Novex, Frankfurt, Germany) with a neutral pH, pre-cast polyacrylamide mini-gel system and NuPAGE MES SDS Running Buffer. An 8ug aliquot of each activated CRM or

10   conjugate was mixed with reducing sample buffer and heated at 100°C for 5 minutes. The conjugates and molecular weight (MW) standards (Invitrogen, Carlsbad, CA) were loaded on a 10% (w/v, acrylamide) NuPage gel (Novex) based upon a Bis-Tris-HCl buffered system and run on MES SDS Running Buffer-PAGE (Laemmli). Following SDS-PAGE, the gel was stained with Pierce Gel Code Blue (Pierce, 15   Rockford, IL). A $\beta$  peptide-CRM<sub>197</sub> conjugate was represented by a major band around 66 kDa, above the band of native CRM and a dimer band around 120 kDa, along with minor multimer bands (data not shown).

15     **III. MALDI-TOF Mass Spectrometry Analysis of Peptide-CRM<sub>197</sub> Conjugates:**

20   Mass spectrometry was used for immediate approximation of the degree of conjugation. Suitable aliquots of activated CRM<sub>197</sub> and conjugate samples were analyzed by MALDI-TOF mass spectrometry using 3,5-dimethoxy-4-hydroxy-cinnamic acid (sinapinic acid) as the matrix. The molecular weight of activated CRM<sub>197</sub> determined by MALDI-TOF mass spectrometry (Finnigan MAT Lasermat 2000 Mass Spectrometer, Ringoes, NY) was found to be centered around 60.5kDa 25   and for conjugates varied from 65kDa to 74kDa depending on the degree of conjugation (data not shown). Up to 22 of the lysines (~50%) in CRM<sub>197</sub> were found to be modified at 1:1 ratio.

25     **IV. Optimization experiments:**

30   The degree of activation and conjugation are a function of reagent:protein ratio, temperature of the reaction and pH of the reaction buffer. Some examples are given below to illustrate the optimal conjugation conditions carried out to identify the

optimal pH conditions in order to have reproducible process control parameters for conjugation reactions. Results (Figure 3) showed that the conjugation reaction to A $\beta$  5mer (DAEFRC) as well as A $\beta$  7mer (DAEFRHDC) is pH dependent and yields a higher degree of modification/conjugation when the pH of the reaction condition is increased. Using the TFA salt of 5mer and 7mer peptides, the degree of conjugation was evaluated at pH 9.0 with varying amounts of peptide load (Figure 4). It is evident from these results that peptide conjugates with a defined number of peptide copies per CRM molecule can be generated by varying the peptide /activated CRM ratio during the conjugation process. Similar experiments were done using acetate salt of A $\beta$  7mer peptide.

For the A $\beta$ 1-7/CRM conjugation, the capping process was evaluated by comparing the moles of CMCA per CRM to the moles of CMC per CRM. Since the total of the CMC and CMCA was constant for each peptide:CRM ratio tested, the capping process was presumed to be complete (Figure 5). The total modification in the conjugate stayed between 19 and 21, comparable to the number of lysines bromoacetylated (Figure 5). These experiments were done with TFA as the counterion for the peptide. The A $\beta$ 1-7/CRM conjugation was repeated using the acetate salt of the peptide rather than the TFA salt, and these data are shown in Figure 5 and 6. The capping process appeared to go to completion, with the total of the CMC and CMCA for each point staying between 20 and 22. The conditions for the A $\beta$ -CRM conjugation reaction have been optimized at pH 9.0, with the degree of conjugation controlled by the peptide to CRM ratio in the reaction. By varying the ratio from 0.1 to 1.5, the degree of conjugation can be varied (Figure 6).

The degree of activation and conjugation are a function of reagent:protein ratio, temperature of the reaction and pH of the reaction buffer. The degree of modification (conjugation) for each conjugate was calculated by subtracting the mass value of activated CRM<sub>197</sub> from the mass value of each conjugate and dividing by the mass of the peptide used to prepare the conjugate. The degree of modification (conjugation) for all of the conjugates is described in the Table 2.

The degree of conjugation was also compared to the values determined by the estimated amount of S-carboxymethylcysteine residues formed per mole of CRM<sub>197</sub> (also shown in Table 2).

5      **Table 2: Degree of Modification: Comparison of MALDI-TOF and AAA data**

Sample	Da (From Mass Spectrometry)	Degree of conjugation (From Mass Spectrometry)	Degree of conjugation (From CMC-Amino Acid Analysis)
CRM <sub>197</sub>	58,408	----	----
BrAc-CRM	60,752	19	----
A $\beta$ 1-7/CRM	74,463	14	15
A $\beta$ 1-7/CRM	72,375	12	14
A $\beta$ 1-5/CRM	75,425	20	21
A $\beta$ 1-5/CRM	71,690	15	18

#### EXAMPLE 6

#### IMMUNOGENICITY STUDIES OF A $\beta$ PEPTIDE CONJUGATES

Peptides spanning N-terminal residues 1-5, 1-7, 1-9, and 1-12 of A $\beta$  (with and without

10 the linker sequence GAGAC) and a peptide corresponding to the N-terminus of A $\beta$  in reverse sequence from amino acid twelve to amino acid one (1-12mer in reverse sequence), each conjugated to CRM<sub>197</sub>, were used to immunize mice along with an unconjugated A $\beta$  1-12 mer peptide in a formulation with STIMULON™ QS-21. Each group of mice was immunized subcutaneously with a dose of either 30  $\mu$ g or 5  $\mu$ g of  
 15 one of the samples formulated with 20  $\mu$ g of the adjuvant STIMULON™ QS-21, at the beginning of the study (week 0) and subsequently at weeks 3 and 6. The study protocol is illustrated in Table 3.

As shown in Table 3, peptides spanning N-terminal residues 1-5, 1-7, 1-9, and 1-12

20 of A $\beta$  (with and without the linker sequence GAGAC) and a peptide corresponding to the N-terminus of A $\beta$  in reverse sequence from amino acid twelve to amino acid one

(1-12mer in reverse) conjugated to CRM<sub>197</sub> were used to immunize mice along with unconjugated A $\beta$  1-12 mer peptide in a formulation with QS-21. Each group of mice was vaccinated subcutaneously with a dose of either 30  $\mu$ g or 5  $\mu$ g of one of the samples formulated with 20  $\mu$ g of the adjuvant QS-21, at the beginning of the study

5 (week 0) and subsequently at weeks 3 and 6. Swiss Webster mice were used for the entire study with 5 mice in each group. Injection volume = 100  $\mu$ l; B = Bleed; V = vaccinate; E = exsanguinate.

Anti-A $\beta$  titers were measured by ELISA against A $\beta$  and CRM<sub>197</sub> as described below. Briefly, Costar 96 well plates (#3591) were coated overnight at room temperature 10 with 2  $\mu$ g/mL A $\beta$ 1-42 in sterile carbonate/bicarbonate buffer, pH 9.6. Plates were emptied and blocked for two hours at room temperature with 200  $\mu$ l/well of 0.05% BSA in 1X PBS/0.05% Tween 20. Blocked plates were emptied and washed with a plate washer containing TBS, 0.1% Brij-35 (without azide) wash buffer. All primary antisera were serially diluted with 0.05% BSA in 1X PBS containing 0.05% Tween 15 20/0.02% Azide and 100  $\mu$ L of each dilution was then transferred to the appropriate plate wells and incubated at room temperature for 2 hours. Plates were then emptied/washed as described above. Alkaline phosphatase conjugated goat anti-mouse IgG secondary antibody from Southern Biotech (city, state) was diluted 1:1000 with 0.05% BSA in PBS containing 0.05% Tween 20/0.02% Azide and 100  $\mu$ L 20 was added to each well and incubated at room temperature for 1 hour. Plates were then emptied/washed as described above and finally incubated at room temperature for 1 hour with 100  $\mu$ L/well of a 1 mg/mL solution of p-nitrophenyl phosphate substrate prepared in diethanolamine/MgCl<sub>2</sub>, pH 9.8. The color development was stopped with the addition of 50  $\mu$ L/well of 3 N NaOH. Plates were read at 405 nM 25 with a 690 nM reference. Endpoint titers were calculated at an O.D. of 0.1 AU.

Table 3: Mouse Immunization Study Protocol

Group Code	Description	Dose (µg)	Wk 0	Wk 3	Wk 6	Wk 8	Wk 13	Wk 16
AE488	CRM1-7 w/o linker	30	B, V	E				
AE489	CRM1-12 with linker	30	B, V	E				
AE490	CRM1-9 with linker	30	B, V	E				
AE491	CRM1-7 with linker	30	B, V	E				
AE492	CRM1-5 w/o linker	30	B, V	E				
AE493	CRM1-9 w/o linker	30	B, V	E				
AE494	CRM1-12 w/o linker	30	B, V	E				
AE495	CRM1-5 with linker	30	B, V	E				
AE496	CRM1-7 w/o linker	5	B, V	E				
AE497	CRM1-12 with linker	5	B, V	E				
AE498	CRM1-9 with linker	5	B, V	E				
AE499	CRM1-7 with linker	5	B, V	E				
AE500	CRM1-5 w/o linker	5	B, V	E				
AE501	CRM1-9 w/o linker	5	B, V	E				

Table 3: Mouse Immunization Study Protocol - Continued

Group Code	Description	Dose (µg)	Wk 0	Wk 3	Wk 6	Wk 8	Wk 13	Wk 16
AE502	CRM1-12 w/o linker	5	B, V	B, V	B, V	B	B	E
AE503	CRM1-5 with linker	5	B, V	B, V	B, V	B	B	E
AE504	CRM <sub>197</sub> C1-6151	30	B, V	B, V	B, V	B	B	E
AE505	CRM <sub>197</sub> C1-6151	5	B, V	B, V	B, V	B	B	E
AE506	CRM/12-1mer	30	B, V	B, V	B, V	B	B	E
AE507	CRM/12-1mer	5	B, V	B, V	B, V	B	B	E
AE508	1-12mer peptide	30	B, V	B, V	B, V	B	B	E
AE509	1-12mer peptide	5	B, V	B, V	B, V	B	B	E
AE510	Ab	30	B, V	B, V	B, V	B	B	E
AE511	Ab	5	B, V	B, V	B, V	B	B	E

**CRM<sub>197</sub> ELISA**

Greiner 96 well plates (#650011) were coated at 37°C for 90 minutes with 5.0 µg/mL (100 µL/well) of CRM<sub>197</sub> in sterile carbonate/bicarbonate buffer, pH 9.6. Plates were emptied and washed with a plate washer containing 1X TBS, 0.1% Brij-35 wash buffer. All primary antisera were serially diluted with 1X PBS containing 0.3% Tween 20/EDTA and 100 µL of each dilution was then transferred to the appropriate plate wells and incubated at 37°C for 1 hour. The plates were then emptied/washed as described above. Alkaline phosphatase conjugated goat anti-mouse IgG secondary antibody from Southern Biotech was diluted 1:1000 with 1X PBS containing 0.05% Tween 20/0.02% Azide and 100 µL was added to each well and incubated at 37°C for 1 hour. Plates were then emptied/washed as described above and finally incubated at room temperature for 1 hour with 100 µL/well of a 1 mg/mL solution of p-nitrophenyl phosphate substrate prepared in diethanolamine/MgCl<sub>2</sub>, pH 9.8. The development was stopped with the addition of 50 µL/well of 3 N NaOH. Plates were read at 405 nM with a 690 nM reference. Endpoint titers were calculated at an O.D. of 0.1 AU.

Tables 4-6 illustrate end point ELISA titers against Aβ. Following primary immunization, all eight conjugates (excluding the negative control) induced measurable anti-Aβ IgG immune responses. However, the 30µg dose, but not the 5µg dose, of Aβ gave a positive response at week 3 following primary immunization. Among all the conjugates, it appears that Aβ 1-7 peptide conjugated without linker elicited as good as or better response than other conjugates studied. At 5µg dose, Aβ 1-5C did better at weeks 8-16. Aβ 1-7C was best at 30µg dose. Analysis of antibody titers after second and third immunization with either 5 or 30µg dose indicate that the maximal immune response to Aβ for most of the conjugates was seen after the second immunization. At least in mice, the third immunization did not appear to enhance the immune response. Aβ peptide however, needed three immunizations with the 30µg dose to reach maximal immune response against the peptide (Table 5). In terms of antibody decay over an extended period of time, the antibody level from the groups immunized with conjugates was reduced by 2 to 3 fold as compared to the highest level within that group. Individual samples from weeks 6 and 8 were analyzed to calculate GMTs against Aβ for each of the group (Table 6) to

see if any conjugate group was substantially better than the others. Statistical analysis of week 6 titers from A $\beta$ 1-5C, A $\beta$  1-7C and A $\beta$  1-9C conjugates indicated that the A $\beta$  1-7 conjugate induced a significantly higher titer. It is also evident from this experiment that the linker sequence GAGAC did not contribute to enhancing the 5 immune response to the peptide.

Table 4

Group	Week 0	Week 3	Week 6	Week 8	Week 13	Week 16
1-5C	<100	14,960	687,691	882,012	625,208	771,828
1-7C	<100	51,253	1,280,181	860,463	520,060	571,043
1-9C	<100	18,615	1,008,872	622,325	348,967	380,755
1-12C	<100	615	132,009	390,624	166,162	184,170
1-5LC	<100	4,999	458,075	454,631	237,573	220,091
1-7LC	<100	17,693	849,170	842,402	446,089	400,536
1-9LC	<100	18,544	1,465,115	1,180,347	571,127	579,477
1-12LC	<100	12,664	908,360	598,867	368,101	316,075
CRM <sub>197</sub>	<100	<100	<100	<100	<100	<100
1-42	<100	<100	<100	<100	<100	<100
1-12	<100	<100	<100	<100	<100	<100
12-1C	<100	<100	<100	<100	<100	<100

Table 4. Weeks 0, 3, 6, 8, 13, and 16 ELISA endpoint titers against A $\beta$  using antiserum from 5  $\mu$ g dose of peptide conjugates spanning varying lengths of the N-terminus of Amyloid A $\beta$  peptide. Ref: Eian hyperimmune polyclonal #592 = 3,073,307. Endpoint at O.D. 0.1 AU. Swiss Webster mice were immunized SC-N with 5  $\mu$ g of above antigens formulated with 20  $\mu$ g STIMULON™ QS-21 at weeks 0, 3, and 6.

Table 5

Group	Week 0	Week 3	Week 6	Week 8	Week 13	Week 16
1-5C	<100	18,150	590,355	332,832	204,645	176,159
1-7C	<100	100,672	1,840,741	647,470	592,638	779,072
1-9C	<100	18,520	1,184,696	713,494	363,459	327,085
1-12C	<100	7,837	1,325,725	1,126,389	681,288	577,604
1-5LC	<100	16,347	469,191	184,077	177,358	164,660
1-7LC	<100	47,866	971,229	462,200	463,466	529,726
1-9LC	<100	59,002	921,544	787,273	405,023	500,468
1-12LC	<100	27,348	697,150	483,320	284,800	397,816
CRM <sub>197</sub>	<100	<100	<100	<100	<100	<100
1-42	<100	160	3,327	109,718	48,646	27,901
1-12	<100	<100	<100	<100	<100	<100
12-1C	<100	<100	<100	<100	<100	<100

Table 5. Weeks 0, 3, 6, 8, 13, and 16 ELISA endpoint titers against A $\beta$  using A $\beta$  conjugates spanning varying lengths of the N-terminus of Amyloid A $\beta$  peptide. Ref: Elan hyperimmune polyclonal #582 = 3,073,307. Endpoint at O.D. 0.1 AU. Swiss Webster mice were immunized SC-N with 30  $\mu$ g of above antigens formulated with 20  $\mu$ g STIMULON™ QS-21 at weeks 0, 3, and 6.

Table 6

Group	Week 6	Week 8
1-5C	237,668 <sup>a</sup>	161,671 <sup>b</sup>
1-7C	1,866,702 <sup>a</sup>	881,146 <sup>b</sup>
1-9C	963,323 <sup>a</sup>	595,414 <sup>b</sup>
1-12C	940,260	955,470
1-5LC	395,553	141,084
1-7LC	516,921	394,521
1-9LC	826,773	562,458
1-12LC	544,768	376,952
1-42	365	4,565

Table 6. Weeks 6 and 8 ELISA endpoint GMTs against A $\beta$  using antisera from 30  $\mu$ g dose of peptide conjugates spanning lengths of the N-terminus of Amyloid-A $\beta$ . Ref: Elan Hyperimmune Polyclonal #592 = 3,073,307. Endpoint at O.D. 0.1 AU. Swiss Webster mice were immunized SC-N with 30  $\mu$ g of above antigens formulated with 20  $\mu$ g STIMULON<sup>TM</sup> QS-21 at weeks 0, 3, and 6

a. Statistical analysis of week 6 titers from 1-5C, 1-7C, and 1-9C using Tukey-Kramer show a statistical difference between 1-5C vs 1-7C only, whereas, analysis using Student's T-test shows a statistical difference between 1-5C vs 1-7C and 1-5C vs 1-9C.

b. Statistical analysis of week 8 titers from 1-5C, 1-7C, and 1-9C does not show a statistical difference among the three groups. However, there appears to be a trend that may indicate a difference between 1-5C vs 1-7C.

15 **PDAPP Mouse Brain Tissue Staining**

The PDAPP brain tissue staining assay provides an indication of the functionality of the A $\beta$  peptide conjugates and/or A $\beta$  1-42 antisera. Serum samples from individual mouse groups were separately analyzed for their ability to recognize PDAPP mouse

20 brain tissue plaques containing amyloid peptide. The results are shown in Table 7A and 7B. With the exception of the A $\beta$  5mer conjugate antisera, there was a dose-related response in recognizing the plaques. Independent of the linker, 30 $\mu$ g conjugate-induced antisera had better reactivity patterns as compared to that of 5 $\mu$ g conjugate antisera. However, with the A $\beta$  5mer conjugate antisera, there seems be

25 similar or better reactivity for the 5 $\mu$ g group. Comparing all these results, it is concluded that conjugates made from A $\beta$  1-5 mer through A $\beta$  1-9 mer are sufficient

in eliciting plaques recognizing immune response in mice and the presence of linker is not essential. The following conclusions can be drawn from this study: (a) All of the peptide conjugates induced high titered antiserum against the carrier protein CRM<sub>197</sub> to equal or slightly higher levels as compared to the unconjugated CRM<sub>197</sub>

5 control (not shown). (b) The conjugates with the GAGAC linker did not enhance immunogenicity or functionality compared to conjugates without the linker. (c) The immunogenicity data and PDAPP brain tissue staining (an initial indication of functional antibody) show that the A $\beta$  1-5mer and A $\beta$  1-7mer conjugates appeared to be the preferred immunogens for further development.

10

**Table 7A.** PDAPP mouse brain tissue staining.

5 µg Dose					
Without Linker			With Linker		
Vaccine	Animal #	PDAPP Staining	Vaccine	Animal #	PDAPP Staining
CRM/ A $\beta$ 1-5	1	+(no diffuse)	CRM/ A $\beta$ 1-5	1	-
	2	++/+++		2	-
	3	++/+++		3	$\pm$
	4	++		4	$\pm$
	5	++		5	$\pm$
CRM/ A $\beta$ 1-7	1	++	CRM/ A $\beta$ 1-7	1	+
	2	++		2	++
	3	++		3	++
	4	++		4	+
	5	++		5	++
CRM/ A $\beta$ 1-9	1	+	CRM/ A $\beta$ 1-9	1	++
	2	+/++		2	++
	3	$\pm$		3	+
	4	$\pm$		4	+
	5	$\pm$		5	+
CRM/ A $\beta$ 1-12	1	-	CRM/ A $\beta$ 1-12	1	+
	2	?		2	+
	3	$\pm$		3	++
	4	-		4	-
	5	$\pm$		5	$\pm$
CRM/ A $\beta$ 12-1mer	1	-	A $\beta$ 42	1	-
	2	-		2	-
	3	$\pm$		3	-
	4	-		4	-
	5	$\pm$		5	-

All antiserum diluted 1:1000 for staining procedure.

Table 7B. PDAPP mouse brain tissue staining.

30 µg Dos					
Without Linker			With Linker		
Vaccine	Animal #	PDAPP Staining	Vaccine	Animal #	PDAPP Staining
CRM/ A $\beta$ 1-5	1	-	CRM/ A $\beta$ 1-5	1	+
	2	+/++		2	-
	3	-		3	-
	4	$\pm$		4	$\pm$
	5	++		5	-
CRM/ A $\beta$ 1-7	1	+/++	CRM/ A $\beta$ 1-7	1	+
	2	++		2	$\pm$ /+
	3	++		3	+/++
	4	++		4	$\pm$ /+
	5	++/+++		5	+/++
CRM/ A $\beta$ 1-9	1	++/+++	CRM/ A $\beta$ 1-9	1	+/++
	2	++		2	++
	3	++		3	++
	4	+		4	$\pm$
	5	+		5	+/++
CRM/ A $\beta$ 1-12	1	-	CRM/ A $\beta$ 1-12	1	+/++
	2	+/++		2	+
	3	+/++		3	-
	4	$\pm$		4	+/++
	5	$\pm$		5	+
CRM/ A $\beta$ 12-1mer	1	-	A $\beta$ 42	1	$\pm$
	2	-		2	-
	3	-		3	-
	4	-		4	-
	5	-		5	-

All antiserum diluted 1:1000 for staining procedure.

**EXAMPLE 7****IMMUNOGENICITY STUDIES IN MONKEYS**

Groups of 6 monkeys received 30 ug of 7mer conjugate (total conjugate) 5 adjuvanted with either STIMULON™ QS-21, alum or RC529 SE formulation at days 0, 29 and 58. Additional groups included were 30 ug 5mer conjugate with either alum (Al(OH)<sub>3</sub>) or RC529 SE, 75 and 300 $\mu$ g of A $\beta$  with STIMULON™ QS-21 as positive controls. Positive controls were immunized every two weeks. At day 36 and 10 64 the anti-A $\beta$  antibody titers were determined (Figures 7 - 9). On day 36, 7mer/CRM conjugates with STIMULON™ QS-21, Alum and RC529 SE elicited GMT titers of 10110, 13330 and 17090 respectively (Figure 7). In contrast, A $\beta$  1-42 plus STIMULON™ QS-21 elicited GMTs of 223 and 1734 at 75 and 300 $\mu$ g dose levels, respectively. The A $\beta$  5mer conjugate elicited a titer of 2134 with alum and 15980 with RC529 SE. On day 64, i.e. after 3 doses of conjugates with either STIMULON™ 15 QS21 or RC-529 SE induced substantially higher titers than post second dose (GMTs 69910 for 7 mer/RC-529 SE; 21640 for A $\beta$  5mer/RC-529 SE and 30310 for A $\beta$  7mer/STIMULON™ QS-21) (Figure 8). Conjugates with alum elicited reduced titers at post third immunization compared to post second immunization. It appears that the A $\beta$  7mer conjugate elicited a better response as compared to the A $\beta$  5mer 20 conjugate. In monkeys, adjuvanting A $\beta$  7mer conjugate with RC-529 SE or STIMULON™ QS-21 elicited the highest response (Figure 9). The response to the A $\beta$  7mer conjugate with alum was moderate and similar to that of 300ug A $\beta$  1-42 with STIMULON™ QS-21.

25 Several conclusions can be drawn from the current example. First, both conjugates are very immunogenic in primate species. Second, the presence of adjuvants in the immunization formulation significantly influences the immune response. Third, except for the aluminum adjuvant, RC-529 SE and STIMULON™ QS-21 enhance the immune response after each dose of immunization at least up to three doses 30 (Figures 9). Overall, A $\beta$  7mer conjugate induced higher antibody response in the presence of 529, followed by STIMULON™ QS-21 (see Figure 9).

**EXAMPLE 8****PREPARATION OF MULTIPLE ANTIGENIC PEPTIDE (MAP) CONJUGATES AND THEIR IMMUNOGENICITY STUDY**

Several methods are available for generating multiple antigenic sites on the carriers. In the previous examples, each antigenic site is separately conjugated to the carrier by defined conjugation and capping chemistries. In this example, multiple antigenic sites are constructed by solid phase synthesis of tandem repeats of A $\beta$ 1-7 mer. Alternatively these tandem repeats can be coupled with T-cell epitopes with or without linking through a lysine core as described elsewhere. These multiple antigenic peptides were synthesized with an additional cysteinyl residue for conjugation to the carrier protein. Peptides containing one repeat unit (1-7), three repeat units (1-7)<sub>3</sub> and five repeat units (1-7)<sub>5</sub> with an additional cysteinyl residue at the carboxyl end were synthesized. These peptides were covalently attached to bromoacetylated CRM overnight through their C-terminal cysteine residues. The reaction was carried out at pH 9.0-9.2 with peptide:CRM ratios added as outlined in Table 8. Bromoacetyl groups, which did not react with peptide, were capped with N-acetylcysteamine. These lots represent conjugates containing one single copy, three tandem copies, and five tandem copies of the A $\beta$ 1-7 peptide conjugated to CRM, respectively. Table 8 briefly outlines the properties of the samples.

20

**Table 8. Multiple Antigenic Peptide (MAP) Conjugate samples**

Conjugate	Peptide:CRM (w/w)	pH of reaction
Ab(1-7),/CRM	0.37	8.99
Ab(1-7),/CRM	1.02	8.95
Ab(1-7),/CRM	1.67	9.17

Peptide load (the average number of A $\beta$  1-7 peptides per carrier) and capping numbers (Table 9) are the numbers of unique amino acids (CMC or CMCA) per carrier as determined by amino acid analysis. The CMC and CMCA values were referenced to lysine.

**Table 9.** Degree of conjugation and capping of each conjugate

CONJUGATE	Peptide load (CMC)	Capping (CMCA)
A $\beta$ (1-7),/CRM	12.5	11.7
A $\beta$ (1-7) $_{\beta}$ /CRM	10.4	15.2
A $\beta$ (1-7) $_{\beta}$ /CRM	9.8	15.9

Swiss-Webster mice (10 per group) were immunized subcutaneously with 1 or 0.1  $\mu$ g A $\beta$ /CRM conjugated peptide. Half of the mice were immunized with the composition 5 formulated with 100  $\mu$ g of the adjuvant Al(OH) $_{\beta}$ , and half were immunized without adjuvant. Immunizations were scheduled at weeks 0 and 3. Bleeds were scheduled for weeks 0, 3, and 6. Serum samples were analyzed for antibody response against A $\beta$ 1-42 mer peptide. The results are shown in Table 10.

**Table 10.** Anti-A $\beta$  endpoint titers for Multiple Antigenic Peptide (MAP) conjugates.

GROUP CODE	SAMPLE DESCRIPTION	ADJUVANT	WK 0 POOL	WK 3 GMT	WK 6 GMT
AG332	1 $\mu$ G A $\beta$ (1-7),/CRM	AL(OH) $_{\beta}$	<100	18,096	100,279
AG333	1 $\mu$ G A $\beta$ (1-7) $_{\beta}$ /CRM	AL(OH) $_{\beta}$	<100	44,911	420,235
AG334	1 $\mu$ G A $\beta$ (1-7) $_{\beta}$ /CRM	AL(OH) $_{\beta}$	<100	27,032	394,488
AG335	0.1 $\mu$ G A $\beta$ (1-7),/CRM	AL(OH) $_{\beta}$	<100	19,350	66,834
AG336	0.1 $\mu$ BG A $\beta$ (1-7) $_{\beta}$ /CRM	AL(OH) $_{\beta}$	<100	13,307	208,272
AG337	0.1 $\mu$ G A $\beta$ (1-7) $_{\beta}$ /CRM	AL(OH) $_{\beta}$	<100	1,196	22,665
AG338	1 $\mu$ G A $\beta$ (1-7),/CRM	NONE	<100	5,273	370,980
AG339	1 $\mu$ G A $\beta$ (1-7) $_{\beta}$ /CRM	NONE	<100	9,299	541,093
AG340	1 $\mu$ G A $\beta$ (1-7) $_{\beta}$ /CRM	NONE	<100	3,100	185,272
AG341	0.1 $\mu$ G A $\beta$ (1-7),/CRM	NONE	<100	340	25,839
AG342	0.1 $\mu$ G A $\beta$ (1-7) $_{\beta}$ /CRM	NONE	<100	128	5,553
AG343	0.1 $\mu$ G A $\beta$ (1-7) $_{\beta}$ /CRM	NONE	<100	668	2,098

All conjugates induced anti-A $\beta$  1-42 antibody titer after primary immunization and the levels were substantially increased after the booster dose. In the absence of aluminum adjuvant, the differences in dose response were evident both at week 3 15 and week 6 bleeds. The higher dose elicited high-titered antibody response.

Aluminum adjuvant elicited substantially higher antibody response at week 3 at both dose levels (0.1 and 1  $\mu$ g) as compared to the unadjuvanted groups. After secondary immunization, conjugates given at 1  $\mu$ g dose elicited 5 to 10 fold increase in antibody levels. At this dose level peptide conjugates with 3 and 5 repeats induced

5 higher antibody response than a single repeat containing conjugate. The titers against the CRM carrier were also determined, and these are listed in Table 11.

**Table 11.** Anti-CRM endpoint titers for Multiple Antigenic Peptide (MAP) Conjugates.

GROUP CODE	SAMPLE DESCRIPTION	ADJUVANT	WK 0 POOL	WK 3 GMT	WK 6 GMT
AG332	1 $\mu$ G A $\beta$ (1-7) <sub>3</sub> /CRM	Al(OH) <sub>3</sub>	<50	10,531	114,602
AG333	1 $\mu$ G A $\beta$ (1-7) <sub>3</sub> /CRM	Al(OH) <sub>3</sub>	<50	4,274	83,065
AG334	1 $\mu$ G A $\beta$ (1-7) <sub>5</sub> /CRM	Al(OH) <sub>3</sub>	<50	1,680	49,320
AG335	0.1 $\mu$ G A $\beta$ (1-7) <sub>5</sub> /CRM	Al(OH) <sub>3</sub>	<50	1,114	13,231
AG336	0.1 $\mu$ G A $\beta$ (1-7) <sub>5</sub> /CRM	Al(OH) <sub>3</sub>	<50	197	1,484
AG337	0.1 $\mu$ G A $\beta$ (1-7) <sub>5</sub> /CRM	Al(OH) <sub>3</sub>	<50	65	222
AG338	1 $\mu$ G A $\beta$ (1-7) <sub>5</sub> /CRM	NONE	<50	35	309
AG339	1 $\mu$ G A $\beta$ (1-7) <sub>5</sub> /CRM	NONE	<50	29	1,085
AG340	1 $\mu$ G A $\beta$ (1-7) <sub>5</sub> /CRM	NONE	<50	29	542
AG341	0.1 $\mu$ G A $\beta$ (1-7) <sub>5</sub> /CRM	NONE	<50	25	55
AG342	0.1 $\mu$ G A $\beta$ (1-7) <sub>5</sub> /CRM	NONE	<50	25	34
AG343	0.1 $\mu$ G A $\beta$ (1-7) <sub>5</sub> /CRM	NONE	<50	29	ND

10 Animals were immunized at weeks 0 and 3 and bled at weeks 0, 3, and 6. Adjuvant: 100  $\mu$ g Al(OH)<sub>3</sub> or none. ND=Not Determined.

Data in Table 11 indicates that the unadjuvanted groups induced very low levels of anti-CRM antibody response at both 1 $\mu$ g as well as 0.1 $\mu$ g dose levels even after two immunizations. However conjugates with aluminum hydroxide adjuvant induced substantial levels of anti-CRM antibody response at 1 $\mu$ g dose and much lower 15 response at 0.1 $\mu$ g dose. In the presence of the adjuvant, CRM titers were highest for the single repeat conjugate, intermediate for the triple repeat conjugate, and lowest for the quintuple repeat conjugate. This is as expected, since the CRM dose per peptide dose is lowest for A $\beta$ (1-7)<sub>5</sub>/CRM, and highest for A $\beta$ (1-7)<sub>5</sub>/CRM. The differences were only statistically significant at week 6 for the 0.1 $\mu$ g dose.

20

The objective of the current invention is to elicit high titered immunogenic response against the antigenic hapten and not necessarily against the carrier protein. Under

certain circumstances it is desirable to elicit optimal immune response against the hapten antigenic determinant with least or no immune response against the carrier protein. For such applications, conjugates with tandem repeats of multiple antigenic determinants with unadjuvanted formulation will serve the need.

5

#### EXAMPLE 9

##### **PREPARATION OF A $\beta$ -PEPTIDE CONJUGATES WITH VARIOUS CARRIER PROTEINS AND THEIR IMMUNOGENECITY**

10 This example compares the immunogenicity of conjugates using six different carrier proteins. The acetate salt of A $\beta$ 1-7 was added to bromoacetylated carriers in a 1:1 ratio by weight at pH 9. All conjugates except A $\beta$ 1-7/rC5ap were capped with N-acetylcysteamine. All of the alternative carriers are recombinant bacterial proteins, including CRM (diphtheria toxoid), recombinant C5a peptidase (rC5ap; cloned from

15 *Streptococcus agalactiae*, includes D130A and S512A mutations), ORFs 1224, 1664, 2452 (all cloned from *Streptococcus pyogenes*), and T367, T858 (each cloned from *Chlamydia pneumoniae*). A summary of the carriers used is found in Table 12. The degree of conjugation and capping of each A $\beta$  1-7 conjugate to these carriers are presented in Table 13.

20 This study showed that the recombinant C5a peptidase conjugate induced higher titers against A $\beta$  than most of the other carriers tested, including CRM. This difference was statistically significant for week 6 titers of groups that received aluminum hydroxide. In addition, the A $\beta$ 1-7/T858 conjugate was significantly more immunogenic than most other conjugates in the absence of adjuvant. The only conjugate that performed poorly relative to the CRM control conjugate was A $\beta$ 1-7/T367, a conjugate that also did not react with an A $\beta$  specific monoclonal antibody by Western blot. This study confirms that numerous other carriers can be successfully used to immunize against the A $\beta$  peptide.

**Table 12.** List of carriers and conjugate properties

CARRIER PROTEIN	MW of carrier (Da)	# of lysines
CRM	58, 408	39
rC5ap	108, 560	85
ORF1224	30, 950	18
ORF1664	31, 270	38
ORF2452	31, 790	29
T367	49, 700	29
T858	37, 190	23

**Table 13.** Degree Of Conjugation And Capping Of Each Conjugate

CONJUGATE	Peptide load (CMC)	Capping (CMCA)
A $\beta$ 1-7/rC5ap	25.9	-
A $\beta$ 1-7/ORF1224	12.8	5.7
A $\beta$ 1-7/ORF1664	13.4	10.8
A $\beta$ 1-7/ORF2452	12.03	10.5
A $\beta$ 1-7/T367	13.2	8.2
A $\beta$ 1-7/T858	5.2	1.7

5

Conjugation results: Peptide load (the average number of A $\beta$ 1-7 peptides per carrier) and capping number are the numbers of unique amino acids (CMC or CMCA) per carrier as determined by amino acid analysis. The CMC and CMCA values were referenced to lysine.

10

**Immunization results.**

The geometric mean titer for each group in this study is listed in Table 14. At week 3, regardless of the presence of adjuvant, A $\beta$ 1-7/rC5ap induced significantly higher anti-A $\beta$  titers than the corresponding conjugates prepared with *Streptococcus pyogenes*

5 ORFs 1224, 1664, 2452, or *Chlamydia pneumoniae* ORFs T367 and T858. At week 3 in the absence of adjuvant, A $\beta$ 1-7/rC5ap was also more immunogenic than all other conjugates except A $\beta$ 1-7/T858. The T858 conjugate without Al(OH)<sub>3</sub> induced higher titers than the ORF1224, ORF1664, ORF2452, and CRM conjugates without adjuvant. The only conjugate that was significantly less immunogenic than A $\beta$ 1-  
10 7/CRM was A $\beta$ 1-7/T367 ( $p < 0.00002$ ). The T367 carrier performed poorly with or without adjuvant at both weeks 3 and 6. At week 6, the rC5ap conjugate with aluminum hydroxide was more immunogenic ( $p < 0.04$ ) than all the other conjugates except A $\beta$  1-7/ORF2452. In the absence of adjuvant, both A $\beta$ 1-7/rC5ap and A $\beta$ 1-  
15 7/T858 induced significantly higher titers than the ORF1224, ORF1664, or T367 conjugates. A $\beta$ 1-7/CRM without aluminum hydroxide induced higher titers than either A $\beta$ 1-7/ORF1664 or A $\beta$ 1-7/T367.

**Table 14.** Anti-A $\beta$ 1-42 endpoint titers.

Group Code	Sample Description	Adjuvant	Wk 0 Pool	Wk 3 GMT	Wk 6 GMT
AG344	5 $\mu$ g A $\beta$ 1-7/CRM	Al(OH) <sub>3</sub>	<100	21,404	54,157
AG345	5 $\mu$ g A $\beta$ 1-7/C5ap	Al(OH) <sub>3</sub>	<100	61,967	402,972
AG346	5 $\mu$ g A $\beta$ 1-7/ORF1224	Al(OH) <sub>3</sub>	<100	10,711	30,084
AG347	5 $\mu$ g A $\beta$ 1-7/ORF1664	Al(OH) <sub>3</sub>	<100	7,188	43,226
AG348	5 $\mu$ g A $\beta$ 1-7/ORF2452	Al(OH) <sub>3</sub>	<100	11,437	109,091
AG349	5 $\mu$ g A $\beta$ 1-7/T367	Al(OH) <sub>3</sub>	<100	321	5,139
AG350	5 $\mu$ g A $\beta$ 1-7/T858	Al(OH) <sub>3</sub>	<100	16,656	33,328
AG351	5 $\mu$ g A $\beta$ 1-7/CRM	NONE	<100	2,615	119,488
AG352	5 $\mu$ g A $\beta$ 1-7/C5ap	NONE	<100	11,858	279,113
AG353	5 $\mu$ g A $\beta$ 1-7/ORF1224	NONE	<100	1,674	18,719
AG354	5 $\mu$ g A $\beta$ 1-7/ORF1664	NONE	<100	119	9,832
AG355	5 $\mu$ g A $\beta$ 1-7/ORF2452	NONE	<100	2,493	76,038
AG356	5 $\mu$ g A $\beta$ 1-7/T367	NONE	<100	50	620
AG357	5 $\mu$ g A $\beta$ 1-7/T858	NONE	<100	28,820	275,202

Animals were immunized at weeks 0 and 3 and bled at weeks 0, 3, and 6. Dose is based on the total amount of conjugate. Adjuvant: 100  $\mu$ g Al(OH)<sub>3</sub> or none.

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CLAIMS

We claim:

1. A method for conjugating a peptide immunogen via a reactive group of an amino acid residue of the peptide immunogen to a protein/polypeptide carrier having one or more functional groups, the method comprising the steps of:
  - 5 (a) derivatizing one or more of the functional groups of the protein/polypeptide carrier or optionally to a polypeptide linker attached to the protein/polypeptide carrier to generate a derivatized carrier with reactive sites;
  - 10 (b) reacting the derivatized protein/polypeptide carrier of step (a) with a reactive group of an amino acid of the peptide immunogen under reaction conditions such that the peptide immunogen is conjugated to the derivatized protein/polypeptide carrier via the functional groups;  - 15 (c) further reacting the conjugate with a capping reagent to inactive free, reactive unreacted functional groups on the derivatized protein/polypeptide carrier, thereby preserving the functionality of the carrier, such that it retains its ability to elicit the desired immune responses against the peptide immunogen that would otherwise not occur without a carrier.
2. The method of claim 1, wherein the carrier is selected from the group consisting of human serum albumin, keyhole limpet hemocyanin (KLH), immunoglobulin molecules, thyroglobulin, ovalbumin, influenza hemagglutinin, PADRE polypeptide, malaria circumsporozoite (CS) protein, hepatitis B surface antigen (HBSAg<sub>19-28</sub>), Heat Shock Protein (HSP) 65, *Mycobacterium tuberculosis*, cholera toxin, cholera toxin mutants with reduced toxicity, diphtheria toxin, CRM<sub>197</sub> protein that is cross-reactive with diphtheria toxin, recombinant Streptococcal C5a peptidase, *Streptococcus pyogenes* ORF1224, *Streptococcus pyogenes* ORF1664, *Streptococcus pyogenes* ORF2452, *Chlamydia pneumoniae* ORF T367, *Chlamydia pneumoniae* ORF T858, Tetanus toxoid, HIV gp120 T1, components recognizing microbial

surface adhesive matrix molecules (MSCRAMMS), growth factors, hormones, cytokines and chemokines.

3. The method according to claim 1, wherein the carrier contains a T-cell epitope.
- 5 4. The method according to claim 3, wherein the carrier is a bacterial toxoid.
5. The method according to claim 3, wherein the carrier is influenza hemagglutinin.
6. The method according to claim 3, wherein the carrier is the PADRE polypeptide.
- 10 7. The method according to claim 3, wherein the carrier is malaria circumsporozite (CS) protein.
8. The method according to claim 3, wherein the carrier is Hepatitis B surface antigen (HBsAg<sub>19-28</sub>).
9. The method according to claim 3, wherein the carrier is heat shock protein 65 (HSP 65).
- 15 10. The method according to claim 3, wherein the carrier is a polypeptide from *Mycobacterium tuberculosis* (BCG).
11. The method according to claim 4, wherein the carrier is tetanus toxoid.
12. The method according to claim 4, wherein the bacterial toxoid is CRM 197.
- 20 13. The method according to claim 3, wherein the carrier is recombinant Streptococcal C5a peptidase.
14. The method according to claim 3, wherein the carrier is *Streptococcus pyogenes* ORF 1224.
15. The method according to claim 3, wherein the carrier is *Streptococcus pyogenes* ORF 1664.
- 25 16. The method according to claim 3, wherein the carrier is *Streptococcus pyogenes* ORF 2452.

17. The method according to claim 3, wherein the carrier is *Chlamydia pneumoniae* ORF T367.
18. The method according to claim 3, wherein the carrier is *Chlamydia pneumoniae* ORF T858.
- 5 19. The method according to claim 1, wherein the carrier is a growth factor or hormone, which stimulates or enhances immune response.
20. The method according to claim 19, wherein the growth factor or hormone is selected from the group consisting of IL-1, IL-2,  $\gamma$ -interferon, IL-10, GM-CSF, MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES.
- 10 21. The method according to claim 1, wherein the peptide immunogen is selected from the group consisting of a bacterial protein, a viral protein, and a eukaryotic protein.
22. The method according to claim 21, wherein the peptide immunogen is derived from a protein antigen from a bacterium.
- 15 23. The method according to claim 22, wherein the bacterium is selected from the group consisting of *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Vibrio cholerae*, *Clostridium perfringens*, *Clostridium botulinum*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Borrelia burgdorferi*, *Shigella flexneri*, *Shigella boydii*, *Shigella dysenteriae*, *Allorococcus otitidis* and Group B *streptococci*.
- 20 24. The method according to claim 21, wherein the peptide immunogen is derived from a protein antigen from a virus.
- 25 25. The method according to claim 24, wherein the virus is selected from the group consisting of human immunodeficiency virus (HIV), herpes simplex virus (HSV), human papilloma virus (HPV), parainfluenza virus (PIV), vesicular stomatitis virus (VSV), respiratory syncytial virus (RSV), Epstein-Barr virus (EBV), coronavirus, vaccinia virus, rotavirus, rabies virus, hepatitis C virus (HCV) and hepatitis B virus (HBV).

26. The method according to claim 21, wherein the peptide immunogen is derived from a protein antigen from a fungus.
27. The method according to claim 26 wherein the fungus is selected from the group consisting of *Candida albicans*, *Cryptococcus neoformans*, *Coccidioides* species, *Histoplasma* species, and *Aspergillus* species.
- 5 28. The method according to claim 21, wherein the peptide immunogen is derived from a protein antigen from a parasite.
29. The method according to claim 28, wherein the parasite is selected from the group consisting of a *Plasmodium*, a *Trypanosome*, a *Schistosome*, and a *Leishmania*.
- 10 30. The method according to claim 21, wherein the peptide immunogen is derived from a protein antigen from a eukaryote.
31. The method according to claim 30, wherein the eukaryote is a human.
32. The method according to claim 31, wherein the peptide immunogen from the *15* human is derived from a malignant tumor.
33. The method according to claim 32, wherein the tumor antigen is selected from the group consisting of a renal cell carcinoma antigen, a breast carcinoma antigen, a carcinoembryonic antigen, a melanoma (MAGE) antigen, and a prostate cancer specific antigen.
- 20 34. The method according to claim 31, wherein the peptide immunogen is from a human A $\beta$  polypeptide.
35. The method according to claim 34, wherein the peptide immunogen is derived from the N-terminal region of A $\beta$  polypeptide.
36. The method according to claim 34, wherein the peptide immunogen is derived *25* from the C-terminal region of A $\beta$  polypeptide.
37. The method according to claim 34, wherein the peptide immunogen is derived from the internal region of A $\beta$  polypeptide.

38. The method according to claim 35, wherein the A $\beta$  peptide immunogen is a fragment of A $\beta$  selected from the group consisting of A $\beta$ 1-5, 1-6, 1-7, 1-10, 1-12, 3-7, 1-3, and 1-4.
39. The method according to claim 34, wherein the A $\beta$  peptide immunogen is a fragment of A $\beta$  selected from the group consisting of A $\beta$  7-11, 3-28, 17-28, 1-28, 25-35, 35-40 and 35-42.
40. The method according to claim 34, wherein one or more fragments of A $\beta$  peptide immunogen are fused to another peptide immunogen.
41. The method according to claim 40, wherein one molecule of an A $\beta$  peptide immunogen is fused at its C-terminus to the N-terminus of a molecule of the same A $\beta$  peptide immunogen.
42. The method according to claim 40, wherein one molecule of an A $\beta$  peptide immunogen is fused at its C-terminus to the C-terminus of another molecule of the same A $\beta$  peptide immunogen.
43. The method according to claim 40, wherein one molecule of an A $\beta$  peptide immunogen is fused at its C-terminus to the C-terminus of a different molecule of an A $\beta$  peptide immunogen.
44. The method according to claim 40, wherein one molecule of an A $\beta$  peptide immunogen, is fused at its N-terminus to the C-terminus of a molecule of a different A $\beta$  peptide immunogen.
45. The method according to claim 41, 42, 43 or 44, wherein the A $\beta$  peptide immunogen is derived from the N-terminal region of A $\beta$  polypeptide, and is selected from the group consisting of A $\beta$ 1-5, 1-6, 1-7, 1-10, 1-12, 3-7, 1-3, and 1-4.
46. The method according to claim 41, 42, 43 or 44, wherein the A $\beta$  peptide immunogen is a fragment of A $\beta$  selected from the group consisting of A $\beta$  7-11, 3-28, 17-28, 1-28, 25-35, 35-40 and 35-42.

47. The method according to claim 40, wherein one or more molecules of an A $\beta$  peptide immunogen are linked to one or more molecules of a heterologous peptide.
48. The method according to claim 47, wherein the A $\beta$  peptide immunogen is derived from the N-terminal region of A $\beta$  polypeptide, and is selected from the group consisting of A $\beta$ 1-5, 1-6, 1-7, 1-10, 1-12, 3-7, 1-3, and 1-4.
49. The method according to claim 47, wherein the A $\beta$  peptide immunogen is a fragment of A $\beta$  selected from the group consisting of A $\beta$  7-11, 3-28, 17-28, 1-28, 25-35, 35-40 and 35-42.
50. The method according to claim 47, wherein the heterologous peptide is selected from the group consisting of a T-cell epitope, a B-cell epitope and combinations thereof.
51. The method according to claim 40, wherein one or more molecules of an A $\beta$  peptide immunogen are linked together in a multiple antigenic peptide (MAP) configuration.
52. The method according to claim 40, wherein one or more molecules of an A $\beta$  peptide immunogen are linked together with one or more molecules of a different A $\beta$  peptide immunogen in a multiple antigenic peptide (MAP) configuration.
53. The method according to claim 51 or 52, wherein the A $\beta$  peptide immunogen is derived from the N-terminal region of A $\beta$  polypeptide, and is selected from the group consisting of A $\beta$ 1-5, 1-6, 1-7, 1-10, 1-12, 3-7, 1-3, and 1-4.
54. The method according to claim 51 or 52, wherein the A $\beta$  peptide immunogen is a fragment of A $\beta$  selected from the group consisting of A $\beta$  7-11, 3-28, 17-28, 1-28, 25-35, 35-40 and 35-42.
55. The method according to claim 1, wherein the functional group of one or more amino acid molecules of the protein/polypeptide carrier or of the optionally attached polypeptide linker is derivatized using a cross-linking reagent.

56. The method of claim 55, wherein the derivatizing reagent is a zero-length cross-linking reagent.
57. The method of claim 55, wherein the derivatizing reagent is a homobifunctional cross-linking reagent.
- 5 58. The method of claim 55, wherein the derivatizing reagent is a heterobifunctional cross-linking reagent.
- 10 59.. The method of claim 55, wherein the protein/polypeptide carrier is reacted with a haloacetylating agent.
60. The method of claim 58, wherein the heterobifunctional reagent is a reagent which reacts with a primary or an  $\epsilon$ -amine functional group of one or more amino acid residues of the protein/polypeptide carrier and a pendant thiol group of one or more amino acid residues of the peptide immunogen.
- 15 61. The method of claim 60, wherein the heterobifunctional reagent is selected from the group consisting of N-succinimidyl bromoacetate, N-succinimidyl-3-(2-pyridyl-thio) propionate (SPDP), and succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC).
62. The method of claim 61, wherein the primary or  $\epsilon$ -amine functional group is lysine.
- 20 63. The method according to claim 60, wherein the pendant thiol group is a cysteine residue of the peptide immunogen.
64. The method according to claim 63, wherein the cysteine residue is localized at the amino-terminus of the peptide immunogen.
65. The method according to claim 63, wherein the cysteine residue is localized at carboxy-terminus of the peptide immunogen.
- 25 66. The method according to claim 63, wherein the cysteine residue is localized internally in the peptide immunogen.
67. The method according to claim 63, wherein the pendant thiol group is generated by a thiolating reagent.

68. The method according to claim 67, wherein the thiolating reagent is N-acetyl homocysteinethio lactone.

69. The method according to claim 1, wherein the capping reagent that is used to inactivate free reactive, functional groups on the activated protein/polypeptide carrier is selected from the reagent group consisting of cysteamine, N-acetylcysteamine, and ethanolamine.

5 70. The method according to claim 1, wherein the capping reagent that is used to inactivate free reactive, functional groups on the activated protein/polypeptide carrier is selected from the reagent group consisting of sodium hydroxide, sodium carbonate, ammonium bicarbonate and ammonia.

10 71. The method of claim 1, wherein the reactive group of the amino acid residue of the peptide immunogen is a free sulphhydryl group.

72. The method of claim 1, wherein one or more of the functional groups are on a linker, which is optionally attached to the protein/polypeptide carrier.

15 73. The method of claim 72, wherein the linker is a peptide linker.

74. The method of claim 73, wherein the peptide linker is polylysine.

75. A method for conjugating a peptide immunogen to a protein/polypeptide carrier having the structure:



20 wherein,

C is a protein/polypeptide carrier and X is a derivatizable functional group of an amino acid residue of the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to the protein/polypeptide carrier, and wherein m is an integer greater than 0, but less than or equal to 85, the method comprising the steps of:

25

5

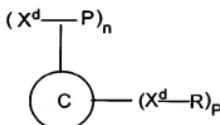
(a). derivatizing one or more of the functional groups of the protein/polypeptide carrier or of the optionally attached linker molecule to generate a derivatized molecule with reactive sites;

(b). reacting the derivatized protein/polypeptide carrier of step (a) with a reactive group of an amino acid residue of the peptide immunogen to form a covalently coupled peptide immunogen- protein/polypeptide carrier conjugate; and

10

(c). further reacting the said conjugate with a capping reagent to inactivate the free reactive functional groups on the activated protein/polypeptide carrier, such that the capped groups are not free to react with other molecules, thereby preserving the functionality of the carrier, such that it retains its ability to elicit the desired immune responses against the peptide immunogen that would otherwise not occur without a carrier, so as to generate a capped peptide immunogen-protein/polypeptide carrier conjugate having the formula:

15



wherein,

20

C is the protein/polypeptide carrier and  $X^d$  is a derivatized functional group of an amino acid residue of the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to the protein/polypeptide carrier, and, wherein,

25

P is a peptide immunogen molecule covalently attached to the derivatized functional group of the amino acid residue of the protein carrier or optionally of an amino acid residue of a peptide linker covalently attached to a protein/polypeptide carrier,

R is a capping molecule covalently attached to the derivatized functional group of an amino acid residue of the protein/polypeptide carrier or

optionally of an amino acid residue of a peptide linker covalently attached to a protein/polypeptide carrier,

n is an integer greater than 0, but less than or equal to 85, and

p is an integer greater than 0, but less than 85.

5 76. The method of claim 75, wherein the carrier is selected from a group consisting of serum albumin, keyhole limpet hemocyanin (KLH), immunoglobulin molecules, thyroglobulin, ovalbumin, influenza hemagglutinin, PADRE polypeptide, malaria circumsporozite (CS) protein, hepatitis B surface antigen (HBSAg<sub>19-28</sub>), Heat Shock Protein (HSP) 65, *Mycobacterium* 10 *tuberculosis*, cholera toxin, cholera toxin mutants with reduced toxicity, diphtheria toxin, CRM<sub>197</sub> protein that is cross-reactive with diphtheria toxin, recombinant Streptococcal C5a peptidase, *Streptococcus pyogenes* 15 ORF1224, *Streptococcus pyogenes* ORF1664, *Streptococcus pyogenes* ORF2452, *Chlamydia pneumoniae* ORF T367, *Chlamydia pneumoniae* ORF T858, Tetanus toxoid, HIV gp120 T1, components recognizing microbial 20 surface adhesive matrix molecules (MSCRAMMS), growth factors, hormones, cytokines and chemokines.

77. The method according to claim 76, wherein the carrier contains a T-cell epitope.

20 78. The method according to claim 77, wherein the carrier is a bacterial toxoid.

79. The method according to claim 77, wherein the carrier is influenza 25 hemagglutinin.

80. The method according to claim 77, wherein the carrier is PADRE polypeptide.

81. The method according to claim 77, wherein the carrier is malaria 30 circumsporozite (CS) protein.

82. The method according to claim 77, wherein the carrier is Hepatitis B surface antigen (HBSAg<sub>19-28</sub>).

83. The method according to claim 77, wherein the carrier is heat shock protein 35 65 (HSP 65).

84. The method according to claim 77, wherein the carrier is a polypeptide from *Mycobacterium tuberculosis* (BCG).
85. The method according to claim 78, wherein the carrier is tetanus toxoid.
86. The method according to claim 78, wherein the bacterial toxoid is CRM 197.
- 5 87. The method according to claim 77, wherein the carrier is Streptococcal rC5a peptidase.
88. The method according to claim 77, wherein the carrier is *Streptococcus pyogenes* ORF1224.
- 10 89. The method according to claim 77, wherein the carrier is *Streptococcus pyogenes* ORF1664.
90. The method according to claim 77, wherein the carrier is *Streptococcus pyogenes* ORF2452.
91. The method according to claim 77, wherein the carrier is *Chlamydia pneumoniae* ORF T367.
- 15 92. The method according to claim 77, wherein the carrier is *Chlamydia pneumoniae* ORF T858.
93. The method according to claim 75, wherein the carrier is a growth factor or hormone, which stimulates or enhances immune response.
- 10 94. The method according to claim 93, wherein the growth factor or hormone is selected from a group consisting of IL-1, IL-2,  $\gamma$ -interferon, IL-10, GM-CSF, MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES.
95. The method according to claim 75, wherein the peptide immunogen is selected from a bacterial protein, a viral protein, and a eukaryotic protein.
- 15 96. The method according to claim 95, wherein the peptide immunogen is derived from a protein antigen from a bacterium.
- 20 97. The method according to claim 96, wherein the bacterium is selected from the group consisting of *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Escherichia coli*, *Klebsiella enterobacter*, *Listeria*

*monocytogenes*, *Vibrio cholerae*, *Clostridium perfringens*, *Clostridium botulinum*, *Pseudomonas* species, *Salmonella typhimurium*, *Borrelia burgdorferi*, *Shigella flexneri*, *Shigella boydii*, *Shigella dysenteriae*, *Alloioococcus otitidis* and Group B streptococci.

- 5 98. The method according to claim 96, wherein the peptide immunogen is derived from a protein antigen from a virus.
99. The method according to claim 98, wherein the virus is selected from the group consisting of human immunodeficiency virus (HIV), herpes simplex virus (HSV), human papilloma virus (HPV), parainfluenza virus (PIV), vesicular stomatitis virus (VSV), respiratory syncytial virus (RSV), Epstein-Barr virus (EBV), coronavirus, vaccinia virus, rotavirus, rabies virus, hepatitis C virus (HCV) and hepatitis B virus (HBV).
- 10 100. The method according to claim 96, wherein the peptide immunogen is derived from a protein antigen from a fungus.
- 15 101. The method according to claim 100 wherein the fungus is selected from the group consisting of *Candida albicans*, *Cryptococcus neoformans*, *Coccidioides*, *Histoplasma*, and *Aspergillus*.
102. The method according to claim 96, wherein the peptide immunogen is derived from a protein antigen from a parasite.
- 20 103. The method according to claim 102, wherein the parasite is selected from the group consisting of *Plasmodium*, a *Trypanosome*, a *Schistosome*, and a *Leishmania*.
104. The method according to claim 96, wherein the peptide immunogen is derived from a protein antigen from a eukaryote.
- 25 105. The method according to claim 104, wherein the eukaryote is a human.
106. The method according to claim 105, wherein peptide immunogen from a human is derived from a malignant tumor.
107. The method according to claim 106, wherein the tumor antigen is a renal cell carcinoma antigen, a breast carcinoma antigen, a carcinoembryonic antigen, a melanoma (MAGE) antigen, and a prostate cancer specific antigen.
- 30

108. The method according to claim 105, wherein the peptide immunogen is from a human A $\beta$  polypeptide.
109. The method according to claim 108, wherein the peptide immunogen is derived from the N-terminal region of A $\beta$  polypeptide.
- 5 110. The method according to claim 108, wherein the peptide immunogen is derived from the C-terminal region of A $\beta$  polypeptide.
111. The method according to claim 108, wherein the peptide immunogen is derived from the internal region of A $\beta$  polypeptide.
- 10 112. The method according to claim 108, wherein the peptide immunogen is a fragment of A $\beta$  selected from the group consisting of A $\beta$ 1-5, 1-6, 1-7, 1-10, 1-12, 3-7, 1-3, and 1-4.
113. The method according to claim 108, wherein the peptide immunogen is a fragment of A $\beta$  selected from the group consisting of A $\beta$  7-11, 3-28, 17-28, 1-28, 25-35, 35-40 and 35-42.
- 15 114. The method according to claim 108, wherein one or more molecules of an A $\beta$  peptide immunogen are fused to another peptide immunogen.
115. The method according to claim 114, wherein one molecule of an A $\beta$  peptide immunogen is fused at its C-terminus to the N-terminus of a another molecule of the same A $\beta$  peptide immunogen.
- 20 116. The method according to claim 114, wherein one molecule of an A $\beta$  peptide immunogen is fused at its C-terminus to the C-terminus of another molecule of the same A $\beta$  peptide immunogen.
117. The method according to claim 114, wherein one molecule of an A $\beta$  peptide immunogen is fused at its C-terminus to the C-terminus of a molecule of a different A $\beta$  peptide immunogen.
- 25 118. The method according to claim 114, wherein one molecule of an A $\beta$  peptide immunogen is fused at its N-terminus to the C-terminus of a molecule of a different A $\beta$  peptide immunogen.

119. The method according to claim 115, 116, 117 or 118, wherein the A $\beta$  fragment is derived from the N-terminal region of the A $\beta$  polypeptide, and is selected from the group consisting of A $\beta$ 1-5, 1-6, 1-7, 1-10, 1-12, 3-7, 1-3, and 1-4.
- 5 120. The method according to claim 115, 116, 117 or 118, wherein the A $\beta$  fragment is a fragment of A $\beta$  selected from A $\beta$  7-11, 3-28, 17-28, 1-28, 25-35, 35-40 and 35-42.
- 10 121. The method according to claim 114, wherein one or more molecules of an A $\beta$  peptide immunogen are fused to one or more molecules of a heterologous peptide.
122. The method according to claim 121, wherein the A $\beta$  peptide immunogen is derived from the N-terminal region of A $\beta$  polypeptide, and is selected from the group consisting of A $\beta$ 1-5, 1-6, 1-7, 1-10, 1-12, 3-7, 1-3, and 1-4.
- 15 123. The method according to claim 121, wherein the A $\beta$  peptide immunogen is a fragment of A $\beta$  selected from the group consisting of A $\beta$  7-11, 3-28, 17-28, 1-28, 25-35, 35-40 and 35-42.
124. The method according to claim 121, wherein the heterologous peptide is selected from the group consisting of a T-cell epitope, a B-cell epitope and combinations thereof.
- 20 125. The method according to claim 114, wherein one or more molecules of an A $\beta$  peptide immunogen are linked together in a multiple antigenic peptide (MAP) configuration.
126. The method according to claim 114, wherein one or more molecules of an A $\beta$  peptide immunogen are linked together with one or more molecules of a different A $\beta$  peptide immunogen in a multiple antigenic peptide (MAP) configuration.
- 25 127. The method according to claim 125 or 126, wherein the A $\beta$  peptide immunogen is derived from the N-terminal region of A $\beta$  polypeptide, and is selected from the group consisting of A $\beta$ 1-5, 1-6, 1-7, 1-10, 1-12, 3-7, 1-3, and 1-4.

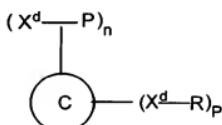
128. The method according to claim 125 or 126, wherein the A $\beta$  peptide immunogen is a fragment of A $\beta$  selected from the group consisting of A $\beta$  7-11, 3-28, 17-28, 1-28, 25-35, 35-40 and 35-42.
129. The method according to claim 75, wherein the functional group of one or more amino acid molecules of the protein/polypeptide carrier or of the optionally attached polypeptide linker is derivatized using a cross-linking reagent.
130. The method of claim 129, wherein the derivatizing reagent is a zero-length cross-linking reagent.
131. The method of claim 129, wherein the derivatizing reagent is a homobifunctional cross-linking reagent.
132. The method of claim 129, wherein the derivatizing reagent is a heterobifunctional cross-linking reagent.
133. The method of claim 129, wherein the protein/polypeptide carrier is reacted with a haloacetylating agent.
134. The method of claim 133, wherein the heterobifunctional reagent is a reagent which reacts with a primary or an  $\epsilon$ -amine functional group of one or more amino acid residues of the protein/polypeptide carrier and a pendant thiol group of one or more amino acid residues of the peptide immunogen.
135. The method of claim 134, wherein the heterobifunctional reagent is N-succinimidyl bromoacetate, N-succinimidyl-3-(2-pyridyl-thio) propionate (SPDP), and succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC).
136. The method of claim 134, wherein the primary or  $\epsilon$ -amine functional group is lysine.
137. The method according to claim 136, wherein derivatization of the primary or  $\epsilon$ -amine functional group of the amino acid residue lysine of the protein/polypeptide carrier with N-succinimidyl bromo acetate results in the bromoacetylation of the primary or  $\epsilon$ -amine residues on lysine residues on the protein/polypeptide carrier.

138. The method according to claim 134, wherein the pendant thiol group is a cysteine residue of the peptide immunogen.
139. The method according to claim 138, wherein the cysteine residue is localized at the amino-terminus of the peptide immunogen.
- 5 140. The method according to claim 138, wherein the cysteine residue is localized at carboxy-terminus of the peptide immunogen.
141. The method according to claim 138, wherein the cysteine residue is localized internally in the peptide immunogen.
142. The method according to claim 138, wherein the pendant thiol group is generated by a thiolating reagent.
- 10 143. The method according to claim 142, wherein the thiolating reagent is N-acetyl homocysteinethio lactone.
144. The method according to claim 75, wherein the capping reagent that is used to inactivate free reactive, functional groups of the activated protein/polypeptide carrier is selected from the reagent group consisting of cysteamine, *N*-acetylcysteamine, and ethanolamine.
- 15 145. The method according to claim 75, wherein the capping reagent that is used to inactivate free reactive, functional groups on the activated protein/polypeptide carrier is selected from the reagent group consisting of sodium hydroxide, sodium carbonate, ammonium bicarbonate and ammonia.
- 20 146. The method of claim 75, wherein the reactive group of the amino acid residue of the peptide immunogen is a free sulphydryl group.
147. The method of claim 75, wherein one or more of the functional groups are on a linker optionally attached to the protein/polypeptide carrier.
- 25 148. The method of claim 147, wherein the linker is a peptide linker.
149. The method of claim 148, wherein the peptide linker is polylysine.
150. A peptide immunogen-protein/polypeptide carrier conjugate wherein the protein/polypeptide carrier has the formula:



wherein,

5                   C is a protein/polypeptide carrier and X is a derivatizable functional group of an amino acid residue on the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to the protein/polypeptide carrier, and, wherein m is an integer greater than 0, but less than or equal to 85, and wherein the peptide immunogen-protein/polypeptide carrier conjugate has the formula:



10                  wherein,

15                  C is the protein/polypeptide carrier and  $X^d$  is a derivatized functional group of an amino acid residue of the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to the protein/polypeptide carrier, and, wherein, P is a peptide immunogen molecule covalently attached to the derivatized functional group of the amino acid residue of the protein carrier or optionally of an amino acid residue of a peptide linker covalently attached to a protein/polypeptide carrier, R is a capping molecule covalently attached to the derivatized functional group of an amino acid residue of the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to a protein/polypeptide carrier, thereby preserving the functionality of the carrier such that it retains its ability to elicit the desired immune responses against the peptide immunogen that would otherwise not occur without a carrier., n is an integer greater than

0, but less than or equal to 85, and p is an integer greater than 0, but less than 85.

151. The conjugate according to claim 150, wherein the carrier is selected from a group consisting of serum albumin, keyhole limpet hemocyanin (KLH), immunoglobulin molecules, thyroglobulin, ovalbumin, influenza hemagglutinin, PADRE polypeptide, malaria circumsporozite (CS) protein, hepatitis B surface antigen (HBsAg<sub>19-28</sub>), Heat Shock Protein (HSP) 65, *Mycobacterium tuberculosis*, cholera toxin, cholera toxin mutants with reduced toxicity, diphtheria toxin, CRM<sub>197</sub> protein that is cross-reactive with diphtheria toxin, recombinant Streptococcal C5a peptidase, *Streptococcus pyogenes* ORF1224, *Streptococcus pyogenes* ORF1664, *Streptococcus pyogenes* ORF2452, *Chlamydia pneumoniae* ORF T367, *Chlamydia pneumoniae* ORF T858, Tetanus toxoid, HIV gp120 T1, components recognizing microbial surface adhesive matrix molecules (MSCRAMMS), growth factors, hormones, cytokines and chemokines.

152. The conjugate according to claim 150, wherein the carrier contains a T-cell epitope.

153. The conjugate according to claim 152, wherein the carrier is a bacterial toxoid.

154. The conjugate according to claim 152, wherein the carrier is influenza hemagglutinin.

155. The conjugate according to claim 152, wherein the carrier is PADRE polypeptide.

156. The conjugate according to claim 152, wherein the carrier is malaria circumsporozite (CS) protein.

157. The conjugate according to claim 152, wherein the carrier is Hepatitis B surface antigen (HBsAg<sub>19-28</sub>).

158. The conjugate according to claim 152, wherein the carrier is heat shock protein 65 (HSP 65).

159. The conjugate according to claim 152, wherein the carrier is a polypeptide from *Mycobacterium tuberculosis* (BCG).
160. The conjugate according to claim 153, wherein the carrier is tetanus toxoid.
161. The conjugate according to claim 153, wherein the bacterial toxoid is CRM<sub>197</sub>.
- 5 162. The conjugate according to claim 152, wherein the carrier is Streptococcal rC5a peptidase.
163. The conjugate according to claim 152, wherein the carrier is *Streptococcus pyogenes* ORF1224.
- 10 164. The conjugate according to claim 152, wherein the carrier is *Streptococcus pyogenes* ORF1664.
165. The conjugate according to claim 152, wherein the carrier is *Streptococcus pyogenes* ORF2452.
166. The conjugate according to claim 152, wherein the carrier is *Chlamydia pneumoniae* ORF T367.
- 15 167. The conjugate according to claim 152, wherein the carrier is *Chlamydia pneumoniae* ORF T858.
168. The conjugate according to claim 150, wherein the carrier is a growth factor or hormone, which stimulates or enhances immune response.
- 20 169. The conjugate according to claim 168, wherein the growth factor or hormone is selected from a group consisting of IL-1, IL-2,  $\gamma$ -interferon, IL-10, GM-CSF, MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES.
170. The conjugate according to claim 150, wherein the peptide immunogen is selected from the group consisting of a bacterial protein, a viral protein, and a eukaryotic protein.
- 25 171. The conjugate according to claim 170, wherein the peptide immunogen is derived from a protein antigen from a bacterium.
172. The conjugate according to claim 171, wherein the bacterium is selected from the group consisting of *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*,

5 *Haemophilus influenzae*, *Esherichia coli*, *Klebsiella enterobacter*, *Listeria monocytogenes*, *Vibrio cholerae*, *Clostridium perfringens*, *Clostridium botulinum*, *Pseudomonas* species, *Salmonella typhimurium*, *Borrelia burgdorferi*, *Shigella flexneri*, *Shigella boydii*, *Shigella dysenteriae*, *Alloiococcus otitidis* and Group B *streptococci*.

10 173. The conjugate according to claim 170, wherein the peptide immunogen is derived from a protein antigen from a virus.

15 174. The conjugate according to claim 173, wherein the virus is selected from the group consisting of a human immunodeficiency virus (HIV), a herpes simplex virus (HSV), a human papilloma virus (HPV), a parainfluenza virus (PIV), a vesicular stomatitis virus (VSV), a respiratory syncytial virus (RSV), an Epstein-Barr virus (EBV), a coronavirus, a vaccinia virus, a rotavirus, a rabies virus, a hepatitis C virus (HCV) and a hepatitis B virus (HBV).

20 175. The conjugate according to claim 170, wherein the peptide immunogen is derived from a protein antigen from a fungus.

25 176. The conjugate according to claim 175, wherein the fungus is selected from the group consisting of *Candida albicans*, *Cryptococcus neoformans*, *Coccidioides*, *Histoplasma*, and *Aspergillus*.

30 177. The conjugate according to claim 170, wherein the peptide immunogen is derived from a protein antigen from a parasite.

178. The conjugate according to claim 177, wherein the parasite is selected from the group consisting of a *Plasmodium*, a *Trypanosome*, a *Schistosome*, and a *Leishmania*.

179. The conjugate according to claim 170, wherein the peptide immunogen is derived from a protein antigen from an eukaryote.

180. The conjugate according to claim 179, wherein the eukaryote is a human.

181. The conjugate according to claim 180, wherein peptide immunogen from the human is derived from a malignant tumor.

35 182. The conjugate according to claim 181, wherein the tumor antigen is selected from the group consisting of a renal cell carcinoma antigen, a breast

carcinoma antigen, a carcinoembryonic antigen, a melanoma (MAGE) antigens, and prostate specific membrane antigen (PSMA).

183. The conjugate according to claim 180, wherein the peptide immunogen is from a human A $\beta$  polypeptide.

5 184. The conjugate according to claim 183, wherein the peptide immunogen is derived from the N-terminal region of A $\beta$  polypeptide.

185. The conjugate according to claim 183, wherein the peptide immunogen is derived from the C-terminal region of A $\beta$  polypeptide.

10 186. The conjugate according to claim 183, wherein the peptide immunogen is derived from the internal region of A $\beta$  polypeptide.

187. The conjugate according to claim 183, wherein the peptide immunogen is a fragment of A $\beta$  selected from the group consisting of A $\beta$ 1-5, 1-6, 1-7, 1-10, 1-12, 3-7, 1-3, and 1-4.

15 188. The conjugate according to claim 183, wherein the peptide immunogen is a fragment of A $\beta$  selected from the group consisting of A $\beta$  7-11, 3-28, 17-28, 1-28, 25-35, 35-40 and 35-42.

189. The conjugate according to claim 183, wherein one or more molecules of A $\beta$  are fused to one or more molecules of another peptide immunogen.

20 190. The conjugate according to claim 189, wherein one molecule of an A $\beta$  peptide immunogen is fused at its C-terminus to the N-terminus of another molecule of the same A $\beta$  peptide immunogen.

191. The conjugate according to claim 189, wherein one molecule of an A $\beta$  peptide immunogen is fused at its C-terminus to the C-terminus of another molecule of the same A $\beta$  peptide immunogen.

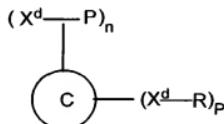
25 192. The conjugate according to claim 189, wherein a molecule of an A $\beta$  peptide immunogen is fused at its C-terminus to the C-terminus of a molecule of a different A $\beta$  peptide immunogen.

193. The conjugate according to claim 189 wherein one molecule of an A $\beta$  peptide immunogen is fused at its N-terminus to the C-terminus of a molecule of a different A $\beta$  peptide immunogen.
194. The conjugate according to claim 190, 191, 192 or 193, wherein the A $\beta$  peptide immunogen is derived from the N-terminal region of the A $\beta$  polypeptide, and is selected from the group consisting of A $\beta$ 1-5, 1-6, 1-7, 1-10, 1-12, 3-7, 1-3, and 1-4.
195. The conjugate according to claim 190, 191, 192 or 193, wherein the A $\beta$  peptide immunogen is a fragment of A $\beta$  selected from the group consisting of A $\beta$  7-11, 3-28, 17-28, 1-28, 25-35, 35-40 and 35-42.
196. The conjugate according to claim 189, wherein one or more molecules of an A $\beta$  peptide immunogen are linked to one or more molecules of a heterologous peptide.
197. The conjugate according to claim 196, wherein the A $\beta$  peptide immunogen is derived from the N-terminal region of A $\beta$  polypeptide, and is selected from the group consisting of A $\beta$ 1-5, 1-6, 1-7, 1-10, 1-12, 3-7, 1-3, and 1-4.
198. The conjugate according to claim 196, wherein the A $\beta$  fragment is a fragment of A $\beta$  selected from the group consisting of A $\beta$  7-11, 3-28, 17-28, 1-28, 25-35, 35-40 and 35-42.
199. The conjugate according to claim 196, wherein the heterologous peptide is selected from the group consisting of a T-cell epitope, a B-cell epitope and combinations thereof.
200. The conjugate according to claim 189, wherein one or more molecules of an A $\beta$  peptide immunogen are linked together in a multiple antigenic peptide (MAP) configuration.
201. The conjugate according to claim 189, wherein one or more molecules of an A $\beta$  peptide immunogen are linked with one or more copies of a different A $\beta$  peptide immunogen in a multiple antigenic peptide (MAP) configuration.

202. The conjugate according to claim 200 or 201, wherein the A $\beta$  peptide immunogen is derived from the N-terminal region of A $\beta$  polypeptide, and is selected from the group consisting of A $\beta$ 1-5, 1-6, 1-7, 1-10, 1-12, 3-7, 1-3, and 1-4.

5 203. The conjugate according to claim 200 or 201, wherein the A $\beta$  peptide immunogen is a fragment of A $\beta$  selected from the group consisting of A $\beta$  7-11, 3-28, 17-28, 1-28, 25-35, 35-40 and 35-42.

204. A peptide immunogen-protein/polypeptide carrier conjugate generated according to the method of claim 75 and having the formula:



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wherein,

15 C is the protein/polypeptide carrier and X<sup>d</sup> is a derivatized functional group of an amino acid residue of the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to the protein/polypeptide carrier, and, wherein, P is a peptide immunogen molecule covalently attached to the derivatized functional group of the amino acid residue of the protein carrier or optionally of an amino acid residue of a peptide linker covalently attached to a protein/polypeptide carrier, R is a capping molecule covalently attached to the derivatized functional group of an amino acid residue of the protein/polypeptide carrier or optionally of an amino acid residue of a peptide linker covalently attached to a protein/polypeptide carrier, which preserves the functionality of the carrier, such that it retains its ability to elicit the desired immune responses against the peptide immunogen that would otherwise not occur without a carrier, n is an integer greater than 0, but less than or equal to 85, and p is an integer greater than 0, but less than 85.

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205. The conjugate according to claim 204 wherein the carrier is selected from the group consisting of human serum albumin, keyhole limpet hemocyanin (KLH), immunoglobulin molecules, thyroglobulin, ovalbumin, influenza hemagglutinin, PADRE polypeptide, malaria circumsporozite (CS) protein, hepatitis B surface antigen (HBSAg<sub>19-28</sub>), Heat Shock Protein (HSP) 65, *Mycobacterium tuberculosis*, cholera toxin, cholera toxin mutants with reduced toxicity, diphtheria toxin, CRM<sub>197</sub> protein that is cross-reactive with diphtheria toxin, recombinant Streptococcal C5a peptidase, *Streptococcus pyogenes* ORF1224, *Streptococcus pyogenes* ORF1664, *Streptococcus pyogenes* ORF2452, *Chlamydia pneumoniae* ORF T367, *Chlamydia pneumoniae* ORF T858, Tetanus toxoid, HIV gp120 T1, components recognizing microbial surface adhesive matrix molecules (MSCRAMMS), growth factors, hormones, cytokines and chemokines.

5 206. The conjugate according to claim 204 wherein the carrier contains a T-cell epitope.

10 207. The conjugate according to claim 206, wherein the carrier is a bacterial toxoid.

15 208. The conjugate according to claim 206, wherein the carrier is influenza hemagglutinin.

20 209. The conjugate according to claim 206, wherein the carrier is PADRE polypeptide.

25 210. The conjugate according to claim 206, wherein the carrier is malaria circumsporozite (CS) protein.

211. The conjugate according to claim 206, wherein the carrier is Hepatitis B surface antigen (HBSAg<sub>19-28</sub>).

25 212. The conjugate according to claim 206, wherein the carrier is heat shock protein 65 (HSP 65).

213. The conjugate according to claim 206, wherein the carrier is a polypeptide from *Mycobacterium tuberculosis* (BCG).

30 214. The conjugate according to claim 207, wherein the carrier is tetanus toxoid.

215. The conjugate according to claim 207, wherein the bacterial toxoid is CRM<sub>197</sub>.
216. The conjugate according to claim 206, wherein the carrier is Streptococcal rC5a peptidase.
217. The conjugate according to claim 206, wherein the carrier is *Streptococcus pyogenes* ORF1224.
- 5 218. The conjugate according to claim 206, wherein the carrier is *Streptococcus pyogenes* ORF1664.
219. The conjugate according to claim 206, wherein the carrier is *Streptococcus pyogenes* ORF2452.
- 10 220. The conjugate according to claim 206, wherein the carrier is *Chlamydia pneumoniae* ORF T367.
221. The conjugate according to claim 206, wherein the carrier is *Chlamydia pneumoniae* ORF T858.
222. The conjugate according to claim 206, wherein the carrier is a growth factor or hormone, which stimulates or enhances immune response.
- 15 223. The conjugate according to claim 222, wherein the growth factor or hormone is selected from a group consisting of IL-1, IL-2,  $\gamma$ -interferon, IL-10, GM-CSF, MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES.
224. The conjugate according to claim 206, wherein the peptide immunogen is selected from the group consisting of a bacterial protein, a viral protein, a fungal protein, a parasite protein and a eukaryotic protein.
- 20 225. The conjugate according to claim 224, wherein the peptide immunogen is derived from a protein antigen from a bacterium.
226. The conjugate according to claim 225, wherein the bacterium is selected from the group consisting of *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Escherichia coli*, *Klebsiella enterobacter*, *Listeria monocytogenes*, *Vibrio cholerae*, *Clostridium perfringens*, *Clostridium botulinum*, *Pseudomonas* species, *Salmonella typhimurium*, *Borrelia*

*burgdorferi*, *Shigella flexneri*, *Shigella boydii*, *Shigella dysentriæ*, *Alloioococcus otitidis* and Group B streptococci.

227. The conjugate according to claim 224, wherein the peptide immunogen is derived from a protein antigen from a virus.

5 228. The conjugate according to claim 227, wherein the virus is selected from the group consisting of a human immunodeficiency virus (HIV), a herpes simplex virus (HSV), a human papilloma virus (HPV), a parainfluenza virus (PIV), a vesicular stomatitis virus (VSV), a respiratory syncytial virus (RSV), Epstein-Barr virus (EBV), a coronavirus, a vaccinia virus, rotavirus, a rabies virus, a hepatitis C virus (HCV) and a hepatitis B virus (HBV).

10 229. The conjugate according to claim 224, wherein the peptide immunogen is derived from a protein antigen from a fungus.

230. The conjugate according to claim 229, wherein the fungus is selected from the group consisting of *Candida albicans*, *Cryptococcus neoformans*, *Coccidioides*, *Histoplasma*, and *Aspergillus*.

15 231. The conjugate according to claim 224, wherein the peptide immunogen is derived from a protein antigen from a parasite.

20 232. The conjugate according to claim 231, wherein the parasite is selected from the group consisting of a *Plasmodium*, a *Trypanosome*, a *Schistosome*, and a *Leishmania*.

233. The conjugate according to claim 224, wherein the peptide immunogen is derived from a protein antigen from an eukaryote.

234. The conjugate according to claim 233, wherein the eukaryote is a human.

25 235. The conjugate according to claim 234, wherein peptide immunogen from the human is derived from a malignant tumor.

236. The conjugate according to claim 235, wherein the tumor antigen is selected from a renal cell carcinoma antigen, a breast carcinoma antigen, a carcinoembryonic antigen, a melanoma antigen, and a prostate cancer specific antigen.

237. The conjugate according to claim 234, wherein the peptide immunogen is from a human A $\beta$  polypeptide.
238. The conjugate according to claim 237, wherein the peptide immunogen is derived from the N-terminal region of A $\beta$  polypeptide.
- 5 239. The conjugate according to claim 237, wherein the peptide immunogen is derived from the C-terminal region of A $\beta$  polypeptide.
240. The conjugate according to claim 237, wherein the peptide immunogen is derived from the internal region of A $\beta$  polypeptide.
- 10 241. The conjugate according to claim 237, wherein the A $\beta$  peptide immunogen is a fragment of A $\beta$  selected from the group consisting of A $\beta$ 1-5, 1-6, 1-7, 1-10, 1-12, 3-7, 1-3, and 1-4.
242. The conjugate according to claim 237, wherein the A $\beta$  peptide immunogen is a fragment of A $\beta$  selected from the group consisting of A $\beta$  7-11, 3-28, 17-28, 1-28, 25-35, 35-40 and 35-42.
- 15 243. The conjugate according to claim 237, wherein one or more molecules of an A $\beta$  peptide immunogen are fused to another peptide immunogen.
244. The conjugate according to claim 243, wherein one molecule of an A $\beta$  peptide immunogen is fused at its C-terminus to the N-terminus of another molecule of the same A $\beta$  peptide immunogen.
- 20 245. The conjugate according to claim 243, wherein one molecule of an A $\beta$  peptide immunogen is fused at its C-terminus to the C-terminus of another molecule of the same A $\beta$  peptide immunogen.
246. The conjugate according to claim 243, wherein one molecule of an A $\beta$  peptide immunogen is fused at its C-terminus to the C-terminus of a molecule of a different A $\beta$  peptide immunogen.
- 25 247. The conjugate according to claim 243 wherein one molecule of an A $\beta$  peptide immunogen is fused at its N-terminus to the C-terminus of a molecule of a different A $\beta$  peptide immunogen.

248. The conjugate according to claim 244, 245, 246 or 247, wherein the A $\beta$  peptide immunogen is derived from the N-terminal region of the A $\beta$  polypeptide, and is selected from the group consisting of A $\beta$ 1-5, 1-6, 1-7,1-10, 1-12, 3-7, 1-3, and 1-4.

5 249. The conjugate according to claim 244, 245, 246 or 247, wherein the A $\beta$  peptide immunogen is a fragment of A $\beta$  selected from the group consisting of A $\beta$  7-11, 3-28, 17-28, 1-28, 25-35, 35-40 and 35-42.

250. The conjugate according to claim 243, wherein one or more fragments of A $\beta$  are linked to one or more molecules of a heterologous peptide.

10 251. The conjugate according to claim 250, wherein the A $\beta$  peptide immunogen is derived from the N-terminal region of A $\beta$  polypeptide, and is selected from the group consisting of A $\beta$ 1-5, 1-6, 1-7,1-10, 1-12, 3-7, 1-3, and 1-4.

252. The conjugate according to claim 250, wherein the A $\beta$  peptide immunogen is a fragment of A $\beta$  selected from the group consisting of A $\beta$  7-11, 3-28, 17-28, 1-28, 25-35, 35-40 and 35-42.

15 253. The conjugate according to claim 250, wherein the heterologous peptide is selected from the group consisting of a T-cell epitope, a B-cell epitope and combinations thereof.

254. The conjugate according to claim 243, wherein one or more molecules of an A $\beta$  peptide immunogen are linked together in a multiple antigenic peptide (MAP) configuration.

20 255. The conjugate according to claim 243, wherein one or more molecules of an A $\beta$  peptide immunogen are linked with one or more copies of a different A $\beta$  peptide immunogen in a multiple antigenic peptide (MAP) configuration.

256. The conjugate according to claim 254 or 255, wherein the A $\beta$  peptide immunogen is derived from the N-terminal region of A $\beta$  polypeptide, and is selected from the group consisting of A $\beta$ 1-5, 1-6, 1-7,1-10, 1-12, 3-7, 1-3, and 1-4.

257. The conjugate according to claim 254 or 255, wherein the A $\beta$  peptide immunogen is a fragment of A $\beta$  selected from the group consisting of A $\beta$  7-11, 3-28, 17-28, 1-28, 25-35, 35-40 and 35-42.

5 258. The conjugate according to claim 243, wherein one or more molecules of an A $\beta$  peptide immunogen are linked with one or more molecules of a heterologous peptide.

259. The conjugate according to claim 258, wherein the heterologous peptide is selected from the group consisting of a T-cell epitope, a B-cell epitope and combinations thereof.

10 260. The conjugate according to claim 258, wherein the A $\beta$  peptide immunogen is derived from the N-terminal region of A $\beta$  polypeptide, and is a fragment of A $\beta$  selected from the group consisting of A $\beta$ 1-5, 1-6, 1-7, 1-10, 1-12, 3-7, 1-3, and 1-4.

15 261. The conjugate according to claim 258, wherein the A $\beta$  peptide immunogen is a fragment of A $\beta$  selected from the group consisting of A $\beta$  7-11, 3-28, 17-28, 1-28, 25-35, 35-40 and 35-42.

20 262. An immunogenic composition, comprising a conjugate of a peptide immunogen with a protein/polypeptide carrier generated by the method of claim 75, together with one or more pharmaceutically acceptable excipients, diluents, and /or adjuvants.

25 263. The immunogenic composition according to claim 262, wherein the carrier is selected from the group consisting of human serum albumin, keyhole limpet hemocyanin (KLH), immunoglobulin molecules, thyroglobulin, ovalbumin, influenza hemagglutinin, PADRE polypeptide, malaria circumsporozite (CS) protein, hepatitis B surface antigen (HBSAg<sub>19-28</sub>), Heat Shock Protein (HSP) 65, *Mycobacterium tuberculosis*, cholera toxin, cholera toxin mutants with reduced toxicity, diphtheria toxin, CRM<sub>197</sub> protein that is cross-reactive with diphtheria toxin, recombinant Streptococcal C5a peptidase, *Streptococcus pyogenes* ORF1224, *Streptococcus pyogenes* ORF1664, *Streptococcus pyogenes* ORF2452, *Chlamydia pneumoniae* ORF T367, *Chlamydia pneumoniae* ORF T858, Tetanus toxoid, HIV gp120 T1, components

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recognizing microbial surface adhesive matrix molecules (MSCRAMMS), growth factors, hormones, cytokines and chemokines.

264. The immunogenic composition according to claim 262, wherein the carrier contains a T-cell epitope.

5 265. The method according to claim 264 wherein the carrier is a bacterial toxoid.

266. The immunogenic composition according to claim 264, wherein the carrier is influenza hemagglutinin.

267. The immunogenic composition according to claim 264, wherein the carrier is PADRE polypeptide.

10 268. The immunogenic composition according to claim 264, wherein the carrier is malaria circumsporozite (CS) protein.

269. The immunogenic composition according to claim 264, wherein the carrier is Hepatitis B surface antigen (HSBAg<sub>19-28</sub>).

270. The immunogenic composition according to claim 264, wherein the carrier is 15 heat shock protein 65 (HSP 65).

271. The immunogenic composition according to claim 264, wherein the carrier is a polypeptide from *Mycobacterium tuberculosis* (BCG).

272. The immunogenic composition according to claim 265, wherein the bacterial toxoid is tetanus toxoid.

20 273. The immunogenic composition according to claim 265, wherein the bacterial toxoid is CRM<sub>197</sub>.

274. The immunogenic composition according to claim 264, wherein the carrier is Streptococcal rC5a peptidase.

275. The immunogenic composition according to claim 264, wherein the carrier is 25 *Streptococcus pyogenes* ORF1224.

276. The immunogenic composition according to claim 264, wherein the carrier is *Streptococcus pyogenes* ORF1664.

277. The immunogenic composition according to claim 264, wherein the carrier is *Streptococcus pyogenes* ORF2452.

278. The immunogenic composition according to claim 264, wherein the carrier is *Chlamydia pneumoniae* ORF T367.

279. The immunogenic composition according to claim 264, wherein the carrier is *Chlamydia pneumoniae* ORF T858.

5 280. The immunogenic composition according to claim 262, wherein the carrier is a growth factor or a hormone, which stimulates or enhances immune response.

281. The immunogenic composition according to claim 280, wherein the growth factor or hormone is selected from a group consisting of IL-1, IL-2, γ-interferon, IL-10, GM-CSF, MIP-1α, MIP-1β, and RANTES.

10 282. The immunogenic composition according to claim 262, wherein the peptide immunogen is selected from the group consisting of a bacterial protein, a viral protein, a fungal protein, a parasite protein, and a eukaryotic protein.

283. The immunogenic composition according to claim 282, wherein the peptide immunogen is derived from a protein antigen from a bacterium.

15 284. The immunogenic composition according to claim 283, wherein the bacterium is selected from the group consisting of *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Escherichia coli*, *Klebsiella* *enterobacter*, *Listeria monocytogenes*, *Vibrio cholerae*, *Clostridium perfringens*, *Clostridium botulinum*, *Pseudomonas* species, *Salmonella* *typhimurium*, *Borrelia burgdorferi*, *Shigella flexneri*, *Shigella boydii*, *Shigella dysenteriae*, *Alloiococcus otitidis* and Group B *streptococci*.

20 285. The immunogenic composition according to claim 282, wherein the peptide immunogen is derived from a protein antigen from a virus.

25 286. The immunogenic composition according to claim 285, wherein the virus is selected from the group consisting of a human immunodeficiency virus (HIV), a herpes simplex virus (HSV), a human papilloma virus (HPV), a parainfluenza virus (PIV), a vesicular stomatitis virus (VSV), a respiratory syncytial virus (RSV), an Epstein-Barr virus (EBV), a coronavirus, a vaccinia

30

virus, a rotavirus, a rabies virus, a hepatitis C virus (HCV) and a hepatitis B virus (HBV).

287. The immunogenic composition according to claim 282, wherein the peptide immunogen is derived from a protein antigen from a fungus.

5 288. The immunogenic composition according to claim 287, wherein the fungus is selected from the group consisting of *Candida albicans*, *Cryptococcus neoformans*, *Coccidioides*, *Histoplasma*, and *Aspergillus*.

289. The immunogenic composition according to claim 282, wherein the peptide immunogen is derived from a protein antigen from a parasite.

10 290. The immunogenic composition according to claim 289, wherein the parasite is selected from the group consisting of a *Plasmodium*, a *Trypanosome*, a *Schistosome*, and a *Leishmania*.

291. The immunogenic composition according to claim 282, wherein the peptide immunogen is derived from a protein antigen from a eukaryote.

15 292. The immunogenic composition according to claim 291, wherein the eukaryote is a human.

293. The immunogenic composition according to claim 292, wherein peptide immunogen from a human is derived from a malignant tumor.

20 294. The immunogenic composition according to claim 293, wherein the peptide immunogen is selected from the group consisting of a renal cell carcinoma antigen, a breast carcinoma antigen, a carcinoembryonic antigen, a melanoma antigen, and a prostate cancer specific antigen.

295. The immunogenic composition according to claim 292, wherein the peptide immunogen is from a human A $\beta$  polypeptide.

25 296. The immunogenic composition according to claim 295, wherein the peptide immunogen is derived from the N-terminal region of A $\beta$  polypeptide.

297. The immunogenic composition according to claim 295, wherein the peptide immunogen is derived from the C-terminal region of A $\beta$  polypeptide.

298. The immunogenic composition according to claim 295, wherein the peptide immunogen is derived from the internal region of A $\beta$  polypeptide.

299. The immunogenic composition according to claim 295, wherein the A $\beta$  peptide immunogen is derived from the N-terminal region of A $\beta$  polypeptide, and is a fragment of A $\beta$  selected from the group consisting of A $\beta$ 1-5, 1-6, 1-7,1-10, 1-12, 3-7, 1-3, and 1-4.

300. The immunogenic composition according to claim 295, wherein the A $\beta$  peptide immunogen is a fragment of A $\beta$  selected from the group consisting of A $\beta$  7-11, 3-28, 17-28, 1-28, 25-35, 35-40 and 35-42.

301. The immunogenic composition according to claim 295, wherein one molecule of an A $\beta$  peptide immunogen is fused at its C-terminus to the N-terminus of another molecule of the same A $\beta$  peptide immunogen.

302. The immunogenic composition according to claim 295, wherein one molecule of an A $\beta$  peptide immunogen is fused at its C-terminus to the C-terminus of another molecule of the same A $\beta$  peptide immunogen.

303. The immunogenic composition according to claim 295, wherein one molecule of an A $\beta$  peptide immunogen is fused at its C-terminus to the C-terminus of a molecule of a different A $\beta$  peptide immunogen.

304. The immunogenic composition according to claim 295, wherein one molecule of an A $\beta$  peptide immunogen is fused at its N-terminus to the C-terminus of a molecule of a different A $\beta$  peptide immunogen.

305. The immunogenic composition according to claim 301, 302, 303, or 304, wherein the A $\beta$  fragment is derived from the N-terminal region of A $\beta$  polypeptide and is selected from the group consisting of A $\beta$ 1-5, 1-6, 1-7,1-10, 1-12, 3-7, 1-3, and 1-4.

306. The immunogenic composition according to claim 301, 302, 303, or 304, wherein the A $\beta$  fragment is a fragment of A $\beta$  selected from the group consisting of A $\beta$  7-11, 3-28, 17-28, 1-28, 25-35, 35-40 and 35-42.

307. The immunogenic composition according to claim 295, wherein one or more molecules of an A $\beta$  peptide immunogen are linked to one or more molecules of a heterologous peptide.

308. The immunogenic composition according to claim 307, wherein the A $\beta$  peptide immunogen is derived from the N-terminal region of A $\beta$  polypeptide, and is selected from the group consisting of A $\beta$ 1-5, 1-6, 1-7, 1-10, 1-12, 3-7, 1-3, and 1-4.

309. The immunogenic composition according to claim 307, wherein the A $\beta$  peptide immunogen is a fragment of A $\beta$  selected from the group consisting of A $\beta$  7-11, 3-28, 17-28, 1-28, 25-35, 35-40 and 35-42.

310. The immunogenic composition according to claim 295, wherein one or more molecules of an A $\beta$  peptide immunogen are linked together in a multiple antigenic peptide (MAP) configuration.

311. The immunogenic composition according to claim 295, wherein one or more molecules of an A $\beta$  peptide immunogen are linked with one or more molecules of a different A $\beta$  peptide immunogen in a multiple antigenic peptide (MAP) configuration.

312. The immunogenic composition according to claim 310 or 311, wherein the A $\beta$  peptide immunogen is derived from the N-terminal region of A $\beta$  polypeptide, and is selected from the group consisting of A $\beta$ 1-5, 1-6, 1-7, 1-10, 1-12, 3-7, 1-3, and 1-4.

313. The immunogenic composition according to claim 310 or 311, wherein the A $\beta$  peptide immunogen is a fragment of A $\beta$  selected from the group consisting of A $\beta$  7-11, 3-28, 17-28, 1-28, 25-35, 35-40 and 35-42.

314. The immunogenic composition according to claim 262, wherein one or more adjuvants are selected from the group consisting of GM-CSF, 529SE, IL-12, aluminum phosphate, aluminum hydroxide, *Mycobacterium tuberculosis*, *Bordetella pertussis*, bacterial lipopolysaccharides, aminoalkyl glucosamine phosphate compounds, MPL<sup>TM</sup> (3-O-deacylated monophosphoryl lipid A), a polypeptide, Quil A, STIMULON<sup>TM</sup> QS-21, a pertussis toxin (PT), an *E.coli*

heat-labile toxin (LT), IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, interferon- $\alpha$ , interferon- $\beta$ , interferon- $\gamma$ , G-CSF, TNF- $\alpha$  and TNF- $\beta$ .

315. The immunogenic composition of claim 314, wherein the peptide immunogen is A $\beta$ , the carrier is CRM<sub>197</sub>, and the adjuvant is 529 SE.

5 316. A method for inducing an immune response in a mammalian subject, which comprises administering an effective amount of the immunogenic composition of claim 262 to the subject.

10 317. The method according to claim 316, wherein the carrier is selected from the group consisting of human serum albumin, keyhole limpet hemocyanin (KLH), immunoglobulin molecules, thyroglobulin, ovalbumin, influenza hemagglutinin, PADRE polypeptide, malaria circumsporozite (CS) protein, hepatitis B surface antigen (HBsAg<sub>19-26</sub>), Heat Shock Protein (HSP) 65, *Mycobacterium tuberculosis*, cholera toxin, cholera toxin mutants with reduced toxicity, diphtheria toxin, CRM<sub>197</sub> protein that is cross-reactive with diphtheria toxin, recombinant Streptococcal C5a peptidase, *Streptococcus pyogenes* ORF1224, *Streptococcus pyogenes* ORF1664, *Streptococcus pyogenes* ORF2452, *Chlamydia pneumoniae* ORF T367, *Chlamydia pneumoniae* ORF T858, Tetanus toxoid, HIV gp120 T1, components recognizing microbial surface adhesive matrix molecules (MSCRAMMS), growth factors, hormones, cytokines and chemokines.

15 318. The method according to claim 316, wherein the carrier contains a T-cell epitope.

20 319. The method according to claim 318, wherein the carrier is a bacterial toxoid.

25 320. The method according to claim 318, wherein the carrier is influenza hemagglutinin.

30 321. The method according to claim 318, wherein the carrier is PADRE polypeptide.

322. The method according to claim 318, wherein the carrier is malaria circumsporozite (CS) protein.

- 323. The method according to claim 318, wherein the carrier is Hepatitis B surface antigen.
- 324. The method according to claim 318, wherein the carrier is heat shock protein 65 (HSP 65).
- 5 325. The method according to claim 318, wherein the carrier is a polypeptide from *Mycobacterium tuberculosis* (BCG).
- 326. The method according to claim 319, wherein the bacterial toxoid is tetanus toxoid.
- 327. The method according to claim 319, wherein the bacterial toxoid is CRM<sub>197</sub>.
- 10 328. The method according to claim 318, wherein the carrier is Streptococcal rC5a peptidase.
- 329. The method according to claim 318, wherein the carrier is *Streptococcus pyogenes* ORF1224.
- 330. The method according to claim 318, wherein the carrier is *Streptococcus pyogenes* ORF1664.
- 15 331. The method according to claim 318, wherein the carrier is *Streptococcus pyogenes* ORF2452.
- 332. The method according to claim 318, wherein the carrier is *Chlamydia pneumoniae* ORF T367.
- 20 333. The method according to claim 318, wherein the carrier is *Chlamydia pneumoniae* ORF T858.
- 334. The method according to claim 316, wherein the carrier is a growth factor or hormone, which stimulates or enhances immune response.
- 25 335. The method according to claim 334, wherein the growth factor or hormone is selected from a group consisting of IL-1, IL-2,  $\gamma$ -interferon, IL-10, GM-CSF, MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES.
- 336. The method according to claim 316, wherein the peptide immunogen is selected from the group consisting of a bacterial protein, a viral protein, and a eukaryotic protein.

337. The method according to claim 336, wherein the peptide immunogen is derived from a protein antigen from a bacterium.

338. The method according to claim 337, wherein the bacterium is selected from *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Escherichia coli*, *Klebsiella enterobacter*, *Listeria monocytogenes*, *Vibrio cholerae*, *Clostridium perfringens*, *Clostridium botulinum*, *Pseudomonas* species, *Salmonella typhimurium*, *Borrelia burgdorferi*, *Shigella flexneri*, *Shigella boydii*, *Shigella dysentriæ*, *Alloioococcus otitidis* and 5 Group B streptococci.

339. The method according to claim 336, wherein the peptide immunogen is derived from a protein antigen from a virus.

340. The method according to claim 339, wherein the virus is selected from the group consisting of a human immunodeficiency virus (HIV), a herpes simplex 15 virus (HSV), a human papilloma virus (HPV), a parainfluenza virus (PIV), a vesicular stomatitis virus (VSV), a respiratory syncytial virus (RSV), a Epstein-Barr virus (EBV), a coronavirus, a vaccinia virus, a rotavirus, a rabies virus, a hepatitis C virus (HCV) and a hepatitis B virus (HBV).

341. The method according to claim 336, wherein the peptide immunogen is 20 derived from a protein antigen from a fungus.

342. The method according to claim 341, wherein the fungus is selected from the group consisting of a *Candida* species, a *Cryptococcus* species, a *Coccidioides* species, a *Histoplasma* species, and an *Aspergillus* species.

343. The method according to claim 336, wherein the peptide immunogen is 25 derived from a protein antigen from a parasite.

344. The method according to claim 343, wherein the parasite is selected from the group consisting of a *Plasmodium*, a *Trypanosome*, a *Schistosome*, and a *Leishmania*.

345. The method according to claim 336, wherein the peptide immunogen is 30 derived from a protein antigen from a eukaryote.

346. The method according to claim 345, wherein the eukaryote is a human.
347. The method according to claim 347, wherein peptide immunogen from a human is derived from a malignant tumor.
- 5 348. The method according to claim 347, wherein the peptide immunogen is derived from a renal cell carcinoma antigen, a breast carcinoma antigen, a carcinoembryonic antigen, a melanoma (MAGE-1) antigen, and a prostate cancer specific antigen.
349. The method according to claim 346, wherein the peptide immunogen is from a human A $\beta$  polypeptide.
- 10 350. The method according to claim 349, wherein the peptide immunogen is derived from the N-terminal region of A $\beta$  polypeptide.
351. The method according to claim 349, wherein the peptide immunogen is derived from the C-terminal region of A $\beta$  polypeptide.
- 15 352. The method according to claim 349, wherein the peptide immunogen is derived from the internal region of A $\beta$  polypeptide.
353. The method according to claim 350, wherein the A $\beta$  peptide immunogen is derived from the N-terminal region of A $\beta$  polypeptide, and is a fragment of A $\beta$  selected from the group consisting of A $\beta$ 1-5, 1-6, 1-7, 1-10, 1-12, 3-7, 1-3, and 1-4.
- 20 354. The method according to claim 349, wherein the A $\beta$  peptide immunogen is a fragment of A $\beta$  selected from the group consisting of A $\beta$  7-11, 3-28, 17-28, 1-28, 25-35, 35-40 and 35-42.
355. The method according to claim 349, wherein one molecule of an A $\beta$  peptide immunogen is fused at its C-terminus to the N-terminus of another molecule 25 of the same A $\beta$  peptide immunogen.
356. The method according to claim 349, wherein one molecule of an A $\beta$  peptide immunogen is fused at its C-terminus to the C-terminus of another molecule of the same A $\beta$  peptide immunogen.

357. The method according to claim 349, wherein one molecule of an A $\beta$  peptide immunogen is fused at its C-terminus to the C-terminus of a molecule of a different A $\beta$  peptide immunogen.
358. The method according to claim 349, wherein one molecule of an A $\beta$  peptide immunogen is fused at its N-terminus to the C-terminus of a molecule of a different A $\beta$  peptide immunogen.
359. The method according to claim 355, 356, 357, or 358, wherein the A $\beta$  peptide immunogen is derived from the N-terminal region of A $\beta$  polypeptide, and is selected from the group consisting of A $\beta$ 1-5, 1-6, 1-7, 1-10, 1-12, 3-7, 1-3, and 1-4.
360. The method according to claim 355, 356, 357, or 358, wherein the A $\beta$  peptide immunogen is a fragment of A $\beta$  selected from the group consisting of A $\beta$  7-11, 3-28, 17-28, 1-28, 25-35, 35-40 and 35-42.
361. The method according to claim 349, wherein one or more molecules of an A $\beta$  peptide immunogen are linked to one or more copies of a heterologous peptide.
362. The method according to claim 361, wherein the A $\beta$  peptide immunogen is derived from the N-terminal region of A $\beta$  polypeptide, and is selected from the group consisting of A $\beta$ 1-5, 1-6, 1-7, 1-10, 1-12, 3-7, 1-3, and 1-4.
363. The method according to claim 361, wherein the A $\beta$  peptide immunogen is selected from the group consisting of A $\beta$  7-11, 3-28, 17-28, 1-28, 25-35, 35-40 and 35-42.
364. The method according to claim 349, wherein one or more molecules of an A $\beta$  peptide immunogen are linked together in a multiple antigenic peptide (MAP) configuration.
365. The method according to claim 349, wherein one or more molecules of an A $\beta$  peptide immunogen are linked with one or more molecules of a different A $\beta$  peptide immunogen in a multiple antigenic peptide (MAP) configuration.

366. The method according to claim 364 or 365, wherein the A $\beta$  peptide immunogen is derived from the N-terminal region of A $\beta$  polypeptide, and is a fragment of A $\beta$  selected from the group consisting of A $\beta$ 1-5, 1-6, 1-7, 1-10, 1-12, 3-7, 1-3, and 1-4.

5 367. The method according to claim 364 or 365, wherein the A $\beta$  peptide immunogen is a fragment of A $\beta$  selected from the group consisting of A $\beta$  7-11, 3-28, 17-28, 1-28, 25-35, 35-40 and 35-42.

368. The method according to claim 262, wherein one or more adjuvants are selected from the group consisting of GM-CSF, 529SE, IL-12, aluminum phosphate, aluminum hydroxide, *Mycobacterium tuberculosis*, *Bordetella pertussis*, bacterial lipopolysaccharides, aminoalkyl glucosamine phosphate compounds, MPL<sup>TM</sup> (3-O-deacylated monophosphoryl lipid A), a polypeptide, Quil A, STIMULON<sup>TM</sup> QS-21, a pertussis toxin (PT), an *E. coli* heat-labile toxin (LT), IL-1  $\alpha$ , IL-1  $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, interferon- $\alpha$ , interferon- $\beta$ , interferon- $\gamma$ , G-CSF, TNF- $\alpha$  and TNF- $\beta$ .

10 369. The method according to claim 368, wherein the peptide immunogen is A $\beta$ , the carrier is CRM<sub>197</sub>, and the adjuvant is 529 SE.

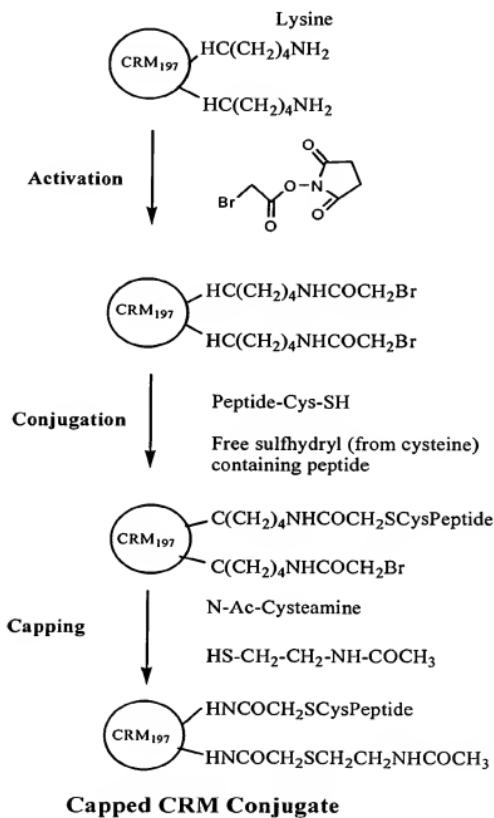
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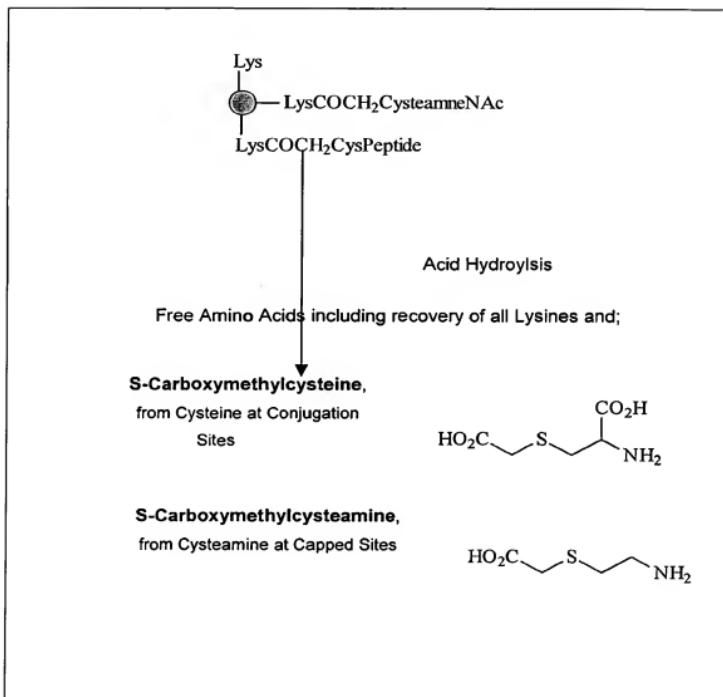
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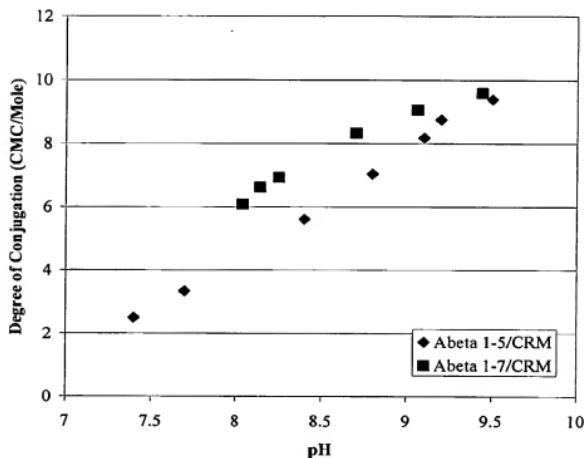
**METHODS OF PRODUCING IMMUNOGENIC PEPTIDE CARRIER CONJUGATES**

**ABSTRACT OF THE DISCLOSURE**

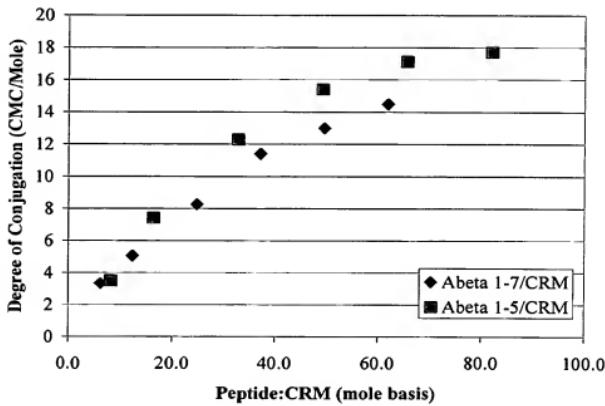
The present invention is directed to methods of producing conjugates of peptide immunogens with protein/polypeptide carrier molecules, which are useful as immunogens, wherein peptide immunogens are conjugated to protein carriers via activated functional groups on amino acid residues of the carrier or of the optionally attached linker molecule, and wherein any unconjugated reactive functional groups on amino acid residues are inactivated via capping, thus retaining the immunological functionality of the carrier molecule, but reducing the propensity for undesirable reactions that could render the conjugate less safe or effective. Furthermore, the invention also relates to such immunogenic products and immunogenic compositions containing such immunogenic products made by such methods

Figure 1. Chemistry for the Preparation of ABetaCRM<sub>197</sub> Conjugate

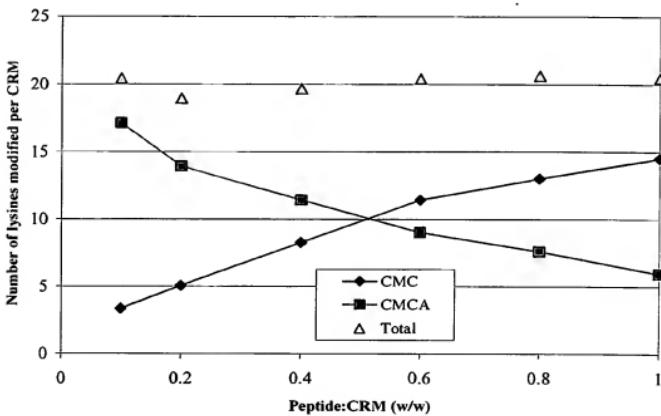
**Figure 2: Acid Hydrolysis of A<sub>β</sub>Peptide-CRM<sub>197</sub> Conjugates.**

**Figure 3: pH dependence of A-beta peptide/CRM conjugation reaction**

**Figure 4:** Dependence of A-beta peptide (TFA salt)/CRM conjugation on peptide:CRM ratio



**Figure 5:** Verification of capping process for  $\text{A}\beta 1-7/\text{CRM}$  conjugation. The pH of the reaction was 9.15. Reaction time with peptide was 16 hrs, capping with *N*-acetylcysteamine was 8 hrs..



**Figure 6:** Conjugation and capping with various peptide:CRM ratios with acetate salt of peptide. The pH of the reaction was 9.0. Reaction time with peptide was 16 hrs, capping with *N*-acetylcysteamine was 8 hrs.

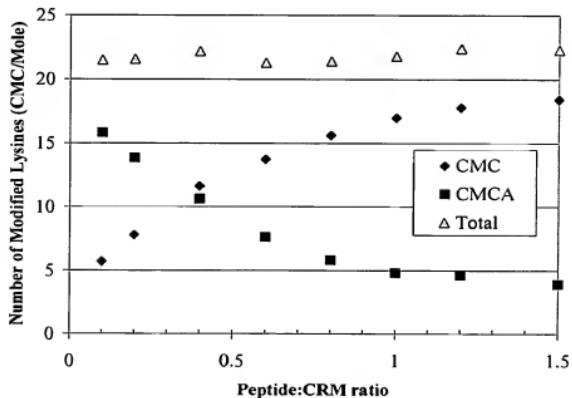


Figure 7: Day 36 titers of primate sera.

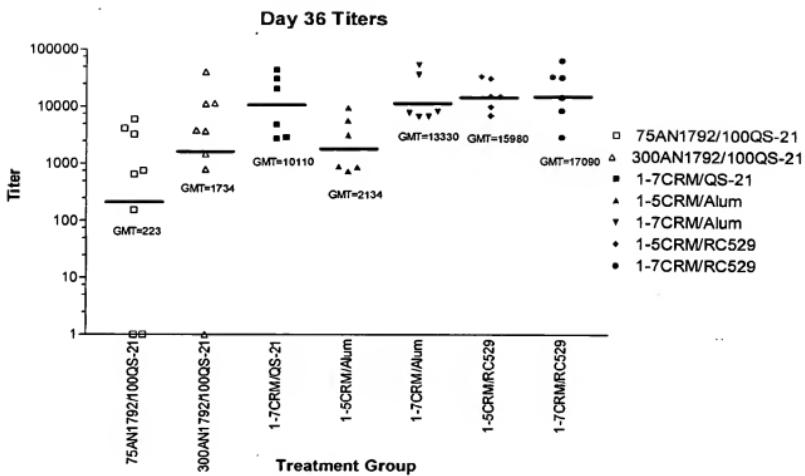
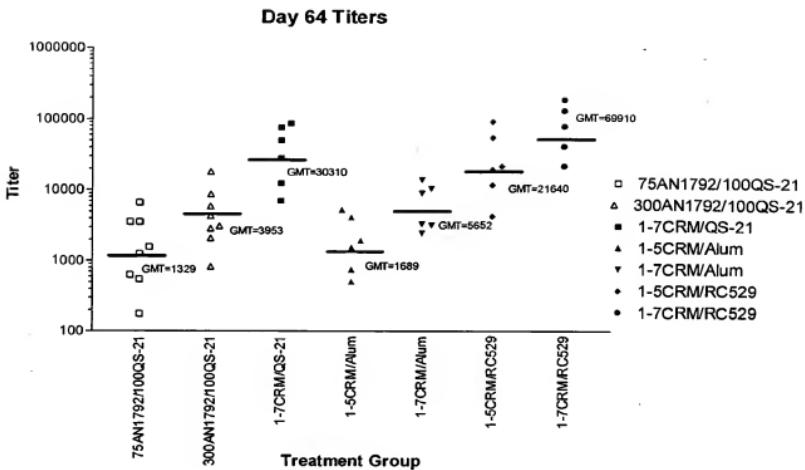
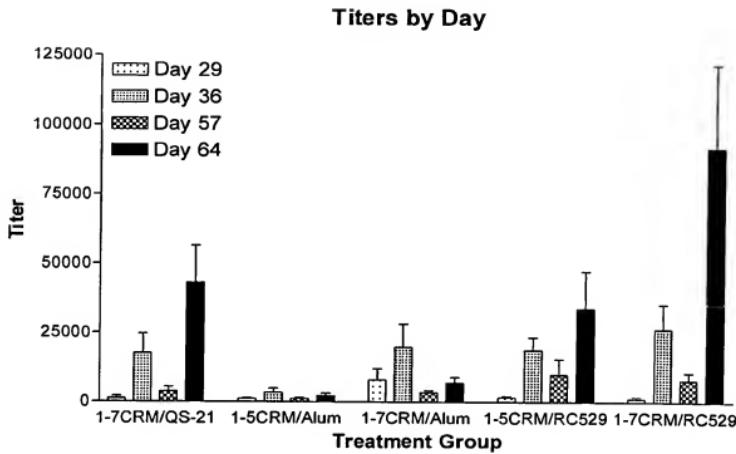


Figure 8: Day 64 titers of primate sera



**Figure 9: Primate titers by day and treatment group**

## SEQUENCE LISTING

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<120> METHODS OF PRODUCING IMMUNOGENIC PEPTIDE CARRIER CONJUGATES

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Ala Ser His Leu Glu

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<210> 19

<211> 15

<212> PRT

<213> Artificial Sequence

<220>

<223> HIV gp120 T1

&lt;400&gt; 19

Lys	Gln	Ile	Ile	Asn	Met	Trp	Gln	Glu	Val	Gly	Lys	Ala	Met	Tyr
1					5				10				15	

&lt;210&gt; 20

&lt;211&gt; 51

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Abeta 1-7/TT830-844/C/TT947-967/Abeta 1-7

&lt;400&gt; 20

Asp	Ala	Glu	Phe	Arg	His	Asp	Gln	Tyr	Ile	Lys	Ala	Asn	Ser	Lys	Phe
1					5				10				15		

Ile	Gly	Ile	Thr	Glu	Leu	Cys	Phe	Asn	Asn	Phe	Thr	Val	Ser	Phe	Trp
		20					25					30			

Leu	Arg	Val	Pro	Lys	Val	Ser	Ala	Ser	His	Leu	Glu	Asp	Ala	Glu	Phe
				35				40				45			

Arg	His	Asp	
	50		

&lt;210&gt; 21

&lt;211&gt; 42

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 21

Asp	Ala	Glu	Phe	Arg	His	Asp	Ser	Gly	Tyr	Glu	Val	His	His	Gln	Lys
1						5			10				15		

Leu	Val	Phe	Phe	Ala	Glu	Asp	Val	Gly	Ser	Asn	Lys	Gly	Ala	Ile	Ile
		20					25				30				

Gly	Leu	Met	Val	Gly	Val	Val	Ile	Ala							
		35				40									

<210> 22  
<211> 22  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> AN90549  
<400> 22

Asp Ala Glu Phe Arg His Asp Gln Tyr Ile Lys Ala Asn Ser Lys Phe  
1 5 10 15

Ile Gly Ile Thr Glu Leu  
20

<210> 23  
<211> 28  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> AN90550  
<400> 23

Asp Ala Glu Phe Arg His Asp Phe Asn Asn Phe Thr Val Ser Phe Trp  
1 5 10 15

Leu Arg Val Pro Lys Val Ser Ala Ser His Leu Glu  
20 25

<210> 24  
<211> 43  
<212> PRT  
<213> Artificial Sequence

<220>

AM100984L1

<223> AN90542

<400> 24

Asp Ala Glu Phe Arg His Asp Gln Tyr Ile Lys Ala Asn Ser Lys Phe  
1 5 10 15

Ile Gly Ile Thr Glu Leu Phe Asn Asn Phe Thr Val Ser Phe Trp Leu  
20 25 30

Arg Val Pro Lys Val Ser Ala Ser His Leu Glu  
35 40

<210> 25

<211> 22

<212> PRT

<213> Artificial Sequence

<220>

<223> AN90576

<400> 25

Glu Phe Arg His Asp Ser Gly Gln Tyr Ile Lys Ala Asn Ser Lys Phe  
1 5 10 15

Ile Gly Ile Thr Glu Leu  
20

<210> 26

<211> 20

<212> PRT

<213> Artificial Sequence

<220>

<223> AN90562

<220>

<221> MISC\_FEATURE

<222> (3)..(3)

<223> X is any amino acid

&lt;400&gt; 26

Ala	Lys	Xaa	Val	Ala	Ala	Trp	Thr	Leu	Lys	Ala	Ala	Ala	Asp	Ala	Glu
1						5				10				15	

Phe Arg His Asp  
20

&lt;210&gt; 27

&lt;211&gt; 34

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; AN90543

&lt;220&gt;

&lt;221&gt; MISC\_FEATURE

&lt;222&gt; (24)...(24)

&lt;223&gt; X is any amino acid

&lt;400&gt; 27

Asp	Ala	Glu	Phe	Arg	His	Asp	Asp	Ala	Glu	Phe	Arg	His	Asp	Asp	Ala
1						5			10				15		

Glu	Phe	Arg	His	Asp	Ala	Lys	Xaa	Val	Ala	Ala	Trp	Thr	Leu	Lys	Ala
						20					25			30	

Ala Ala

&lt;210&gt; 28

&lt;211&gt; 34

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

AM100984L1

<220>  
<223> PADRE/(Ab1-7) X 3  
<220>  
<221> MISC\_FEATURE  
<222> (3)..(3)  
<223> X is any amino acid

<400> 28

Ala Lys Xaa Val Ala Ala Trp Thr Leu Lys Ala Ala Ala Asp Ala Glu  
1 5 10 15

Phe Arg His Asp Asp Ala Glu Phe Arg His Asp Asp Ala Glu Phe Arg  
20 25 30

His Asp

<210> 29  
<211> 20  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> (Ab1-7) X 3/PADRE  
<220>  
<221> MISC\_FEATURE  
<222> (10)..(10)  
<223> X is any amino acid

<400> 29

Asp Ala Glu Phe Arg His Asp Ala Lys Xaa Val Ala Ala Trp Thr Leu  
1 5 10 15

Lys Ala Ala Ala  
20

<210> 30  
<211> 24  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Ab1-7/albumin fragment  
<400> 30

Asp Ala Glu Phe Arg His Asp Ile Ser Gln Ala Val His Ala Ala His  
1 5 10 15

Ala Glu Ile Asn Glu Ala Gly Arg  
20

<210> 31  
<211> 24  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Ab4-10/albumin fragment  
<400> 31

Phe Arg His Asp Ser Gly Tyr Ile Ser Gln Ala Val His Ala Ala His  
1 5 10 15

Ala Glu Ile Asn Glu Ala Gly Arg  
20

<210> 32  
<211> 24  
<212> PRT  
<213> Artificial Sequence

AM100984L1

<220>

<223> Ab3-9/albumin fragment

<400> 32

Glu Phe Arg His Asp Ser Gly Ile Ser Gln Ala Val His Ala Ala His  
1 5 10 15

Ala Glu Ile Asn Glu Ala Gly Arg  
20

<210> 33

<211> 34

<212> PRT

<213> Artificial Sequence

<220>

<223> HA(307-319)/(Ab 1-7) x 3

<400> 33

Pro Lys Tyr Val Lys Gln Asn Thr Leu Lys Leu Ala Thr Asp Ala Glu  
1 5 10 15

Phe Arg His Asp Asp Ala Glu Phe Arg His Asp Asp Ala Glu Phe Arg  
20 25 30

His Asp

<210> 34

<211> 27

<212> PRT

<213> Artificial Sequence

<220>

<223> (Ab 1-7)/HA(307-319)/(Ab 1-7)

<400> 34

Asp Ala Glu Phe Arg His Asp Pro Lys Tyr Val Lys Gln Asn Thr Leu  
1 5 10 15

Lys Leu Ala Thr Asp Ala Glu Phe Arg His Asp  
20 25

<210> 35

<211> 34

<212> PRT

<213> Artificial Sequence

<220>

<223> (Ab 1-7) x 3/HA 307-319

<400> 35

Asp Ala Glu Phe Arg His Asp Asp Ala Glu Phe Arg His Asp Asp Ala  
1 5 10 15

Glu Phe Arg His Asp Pro Lys Tyr Val Lys Gln Asn Thr Leu Lys Leu  
20 25 30

Ala Thr

<210> 36

<211> 27

<212> PRT

<213> Artificial Sequence

<220>

<223> (Ab 1-7) x 2/HA 307-319

<400> 36

Asp Ala Glu Phe Arg His Asp Asp Ala Glu Phe Arg His Asp Pro Lys  
1 5 10 15

Tyr Val Lys Gln Asn Thr Leu Lys Leu Ala Thr  
20 25

<210> 37

AM100984L1

<211> 79

<212> PRT

<213> Artificial Sequence

<220>

<223> Ab 1-7/HA(307-319)/Malaria CS/TT830-844/TT947-967Ab 1-7

<400> 37

Asp Ala Glu Phe Arg His Asp Pro Lys Tyr Val Lys Gln Asn Thr Leu  
1 5 10 15

Lys Leu Ala Thr Glu Lys Lys Ile Ala Lys Met Glu Lys Ala Ser Ser  
20 25 30

Val Phe Asn Val Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile  
35 40 45

Thr Glu Leu Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro  
50 55 60

Lys Val Ser Ala Ser His Leu Glu Asp Ala Glu Phe Arg His Asp  
65 70 75

<210> 38

<211> 58

<212> PRT

<213> Artificial Sequence

<220>

<223> (Ab 1-7) x 3/TT 830-844/TT 947-967

<400> 38

Asp Ala Glu Phe Arg His Asp Asp Ala Glu Phe Arg His Asp Asp Ala  
1 5 10 15

Glu Phe Arg His Asp Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly  
20 25 30

Ile Thr Glu Leu Cys Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg  
35 40 45

Val Pro Lys Val Ser Ala Ser His Leu Glu  
50 55

<210> 39

<211> 44

<212> PRT

<213> Artificial Sequence

<220>

<223> Ab 1-7/TT830-844/C/TT947-967

<400> 39

Asp Ala Glu Phe Arg His Asp Gln Tyr Ile Lys Ala Asn Ser Lys Phe  
1 5 10 15

Ile Gly Ile Thr Glu Leu Cys Phe Asn Asn Phe Thr Val Ser Phe Trp  
20 25 30

Leu Arg Val Pro Lys Val Ser Ala Ser His Leu Glu  
35 40

<210> 40

<211> 535

<212> PRT

<213> Artificial Sequence

<220>

<223> CRM197

<400> 40

Gly Ala Asp Asp Val Val Asp Ser Ser Lys Ser Phe Val Met Glu Asn  
1 5 10 15

Phe Ser Ser Tyr His Gly Thr Lys Pro Gly Tyr Val Asp Ser Ile Gln  
20 25 30

Lys Gly Ile Gln Lys Pro Lys Ser Gly Thr Gln Gly Asn Tyr Asp Asp  
35 40 45

Asp	Trp	Lys	Glu	Phe	Tyr	Ser	Thr	Asp	Asn	Lys	Tyr	Asp	Ala	Ala	Gly
50						55					60				
Tyr Ser Val Asp Asn Glu Asn Pro Leu Ser Gly Lys Ala Gly Gly Val															
65				70			75				80				
Val Lys Val Thr Tyr Pro Gly Leu Thr Lys Val Leu Ala Leu Lys Val															
	85				90			95							
Asp Asn Ala Glu Thr Ile Lys Lys Glu Leu Gly Leu Ser Leu Thr Glu															
100				105			110								
Pro Leu Met Glu Gln Val Gly Thr Glu Glu Phe Ile Lys Arg Phe Gly															
115				120			125								
Asp Gly Ala Ser Arg Val Val Leu Ser Leu Pro Phe Ala Glu Gly Ser															
130				135			140								
Ser Ser Val Glu Tyr Ile Asn Asn Trp Glu Gln Ala Lys Ala Leu Ser															
145				150			155				160				
Val Glu Leu Glu Ile Asn Phe Glu Thr Arg Gly Lys Arg Gly Gln Asp															
165				170			175								
Ala Met Tyr Glu Tyr Met Ala Gln Ala Cys Ala Gly Asn Arg Val Arg															
180				185			190								
Arg Ser Val Gly Ser Ser Leu Ser Cys Ile Asn Leu Asp Trp Asp Val															
195				200			205								
Ile Arg Asp Lys Thr Lys Thr Lys Ile Glu Ser Leu Lys Glu His Gly															
210				215			220								
Pro Ile Lys Asn Lys Met Ser Glu Ser Pro Asn Lys Thr Val Ser Glu															
225				230			235				240				
Glu Lys Ala Lys Gln Tyr Leu Glu Glu Phe His Gln Thr Ala Leu Glu															
245				250			255								
His Pro Glu Leu Ser Glu Leu Lys Thr Val Thr Gly Thr Asn Pro Val															
260				265			270								
Phe Ala Gly Ala Asn Tyr Ala Ala Trp Ala Val Asn Val Ala Gln Val															
275				280			285								
Ile Asp Ser Glu Thr Ala Asp Asn Leu Glu Lys Thr Thr Ala Ala Leu															

290

295

300

Ser Ile Leu Pro Gly Ile Gly Ser Val Met Gly Ile Ala Asp Gly Ala  
 305 310 315 320

Val His His Asn Thr Glu Glu Ile Val Ala Gln Ser Ile Ala Leu Ser  
 325 330 335

Ser Leu Met Val Ala Gln Ala Ile Pro Leu Val Gly Glu Leu Val Asp  
 340 345 350

Ile Gly Phe Ala Ala Tyr Asn Phe Val Glu Ser Ile Ile Asn Leu Phe  
 355 360 365

Gln Val Val His Asn Ser Tyr Asn Arg Pro Ala Tyr Ser Pro Gly His  
 370 375 380

Lys Thr Gln Pro Phe Leu His Asp Gly Tyr Ala Val Ser Trp Asn Thr  
 385 390 395 400

Val Glu Asp Ser Ile Ile Arg Thr Gly Phe Gln Gly Glu Ser Gly His  
 405 410 415

Asp Ile Lys Ile Thr Ala Glu Asn Thr Pro Leu Pro Ile Ala Gly Val  
 420 425 430

Leu Leu Pro Thr Ile Pro Gly Lys Leu Asp Val Asn Lys Ser Lys Thr  
 435 440 445

His Ile Ser Val Asn Gly Arg Lys Ile Arg Met Arg Cys Arg Ala Ile  
 450 455 460

Asp Gly Asp Val Thr Phe Cys Arg Pro Lys Ser Pro Val Tyr Val Gly  
 465 470 475 480

Asn Gly Val His Ala Asn Leu His Val Ala Phe His Arg Ser Ser Ser  
 485 490 495

Glu Lys Ile His Ser Asn Glu Ile Ser Ser Asp Ser Ile Gly Val Leu  
 500 505 510

Gly Tyr Gln Lys Thr Val Asp His Thr Lys Val Asn Ser Lys Leu Ser  
 515 520 525

Leu Phe Phe Glu Ile Lys Ser  
 530 535

<210> 41

<211> 17

<212> PRT

<213> Gallus gallus

<400> 41

Ile Ser Gln Ala Val His Ala Ala His Ala Glu Ile Asn Glu Ala Gly  
1 5 10 15

Arg

## Application Data Sheet

### **Application Information**

Application Type:: Provisional  
Subject Matter:: Utility  
Suggested Classification::  
Suggested Group Art Unit:: TBD  
CD-ROM or CD-R?::  
Number of CD disks:: 1  
Number of copies of CDs:: 1  
Sequence submission?:: Yes  
Computer Readable Form (CRF)?:: Yes  
Number of copies of CRF::  
Title:: METHODS OF PRODUCING IMMUNOGENIC  
PEPTIDE CARRIER CONJUGATES  
Attorney Docket Number:: AM100984L1  
Request for Early Publication?::  
Request for Non-Publication?::  
Suggested Drawing Figure::  
Total Drawing Sheets::

### **Applicant Information**

Applicant Authority Type:: Inventor  
Primary Citizenship Country:: USA  
Given Name:: Rasappa  
Middle Name:: G.  
Family Name:: ARUMUGHAM  
City of Residence:: Chapel Hill  
State or Province of Residence:: North Carolina  
Country of Residence:: USA  
Street of mailing address:: 102 Barton Lane  
City of mailing address:: Chapel Hill  
State or Province of mailing address:: North Carolina  
Country of mailing address:: USA  
Postal or Zip Code of mailing address:: 27516

**Applicant Information**

Applicant Authority Type:: Inventor  
Primary Citizenship Country:: India  
Given Name:: A. Krishna  
Middle Name::  
Family Name:: PRASAD  
City of Residence:: Chapel Hill  
State or Province of Residence:: North Carolina  
Country of Residence:: USA  
Street of mailing address:: 105 Worsham Drive  
City of mailing address:: Chapel Hill  
State or Province of mailing address:: North Carolina  
Country of mailing address:: USA  
Postal or Zip Code of mailing address:: 27516

**Correspondence Information**

Correspondence Customer Number:: 25291  
Phone:: 845 602 4636  
Fax Number:: 845 602 5666  
E-Mail address:: gordona@wyeth.com

**Representative Information**

Representative Customer Number::	25291
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**Domestic Priority Information**

Application::	Continuity Type::	Parent Application::	Parent Filing Date::
This Application			

**Foreign Priority Information**

Country::	Application Number::	Filing Date::	Priority Claimed::

**Assignee Information**

Assignee Name:: Wyeth  
Street of mailing address:: Five Giralta Farms  
City of mailing address:: Madison  
State or Province of mailing address:: New Jersey  
Country of mailing address:: United States of America  
Postal or Zip Code of mailing address:: 07940